Nitric oxide and cyclic nucleotide signalling during the development of the enteric nervous system of the grasshopper, *Locusta migratoria* L.

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Abstract

Neuronal migration is central to the development of nervous systems. In the developing nervous system, most neurons are generated at different sites from those in which they permanently reside (Rakic, 1990). Thus, directed cell migration is necessary to bring these neurons to their ultimate destination (Hatten 1999). The dynamic regulation of nitric oxide synthase (NOS) and its effector enzyme soluble guanylyl cyclase (sGC) during the formation of the nervous system has lead to the suggestion that NO and cyclic nucleotide signalling plays a functional role in the development of nervous systems. Here I investigated an example of neuronal migration in the enteric nervous system (ENS) of the grasshopper. I focus on the directed migration of one set of enteric neurons, the midgut plexus neurons (MG neurons), which undergo a rapid phase of migration, during which the neurons migrate several hundred µm posteriorly on the midgut surface. The MG neurons exhibit NO-induced cGMP-immunoreactivity throughout the phase of migration. Moreover, I identified potential sources of NO near the MG neurons, in a subset of the midgut cells. To investigate a potential role of the NO/cGMP signalling system during the development of the midgut plexus, I examined the pattern of migrating MG neurons in embryo culture. Pharmacological inhibition of endogenous NOS, sGC, and PKG activity results in a significant reduction of MG neuron migration. This pharmacological perturbation of MG neuron migration can be rescued by supplementing with membrane-permeant cGMP and protoporphyrin IX free acid, an activator of sGC, indicating that NO/cGMP signalling is essential for MG neuron migration. Moreover I found that cyclic AMP (cAMP) and protein kinase A (PKA) signalling inhibits migration, indicating that cAMP/PKA signalling has an antagonistic effect on MG neuron migration. In conclusion, both the immunocytochemical staining for cGMP and the pharmacological experiments in embryo culture are consistent with the hypothesis that NO/cGMP/PKG and cAMP/PKA signalling regulate MG neuron migration in the ENS of the grasshopper embryo.

Key words: Nitric oxide/cyclic GMP, signal transduction pathway, neuronal migration

Zusammenfassung

In der vorliegende Dissertation wird die Bedeutung von zwei Signaltransduktionswegen, des Stickstoffmonoxid/zyklisches GMP/Proteinkinase G (NO/cGMP/PKG) und zyclisches AMP/Proteinkinase A (cAMP/PKA) Signalsystem in der Entwicklung des Enterischen Nervensystems des Heuschrecken-Embryos untersucht. Die Arbeit befasst sich mit der Migration von Mitteldarm-Neuronen im Enterischen Nervensystem, die im Laufe der Embryonalentwicklung einen den Mitteldarm umspannenden Nervenplexus bilden. Während der Embryonalentwicklung wandern die Mitteldarm-Neurone in Richtung mehrere hundert µm auf der Darmoberfläche entlang. Die Mitteldarm-Neurone zeigen während ihrer Wanderung NO-induzierte cGMP-Immunoreaktivität (cGMP-IR). Darüber hinaus wurde in unmittelbarer Nähe der migrierenden Neurone eine potentielle NO-Quelle identifiziert. Eine Teilpopulation der Mitteldarm-Zellen zeigt NADPH-diaphorase Färbung, eine histochemischer Marker für NOS-Aktivität. Um die Rolle von NO/cGMP/PKG G und cAMP/PKA in der Entwicklung des Mitteldarm-Plexus zu bestimmen, habe ich das Migrationsverhalten der Mitteldarm-Neurone in Embryo-Kultur untersucht. Die pharmakologische Inhibition von endogener NOS, sGC und PKG führt zu einer signifikanten Reduktion der Migration der Mitteldarm-Neurone. Die migrations-hemmenden Effekte der NOS- bzw. sGC-Inhibitoren konnten durch gleichzeitige Verabreichung von membran-permeablen cGMP bzw. durch die Gabe eines direkten Aktivator der sGC revertiert werden. Darüber hinaus konnte gezeigt werden, das der cAMP/PKA Signalweg eine antagoistische Wirkung auf die Migration der Neurone ausübt. Sowohl ein cAMP-Donor als auch PKA-Aktivator hemmt die Wanderung der Mitteldarm-Neurone. Das Aktin-Zytoskelett der Neurone wurde in einem kombinierten Embryo-Kultur/Zellkultur-Ansatz untersucht. Pharmaka, welche die Migration der Neurone hemmen, bewirken eine Reorganisation des Aktin-Zytoskeletts. Diese pharmakologischen Experimente zeigen, unterstützt durch die immunhistochemischen und histochemischen Ergebnissen, dass das NO/cGMP Signalsystem notwendig für die Migration von Mitteldarm-Neuronen ist und der cAMP/PKA Signaltransduktions Weg einen antagonistischen, hemmenden Effekt auf die Migration ausübt.

Schlagworte: Stickstoffmonoxid/zyclisches GMP, Signalltransduktion, neuronale Migration

Eidesstattliche Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbstständig verfasst habe. Es wurden keine anderen als die angegebenen Quellen und Hilfsmittel verwendet. Diese Dissertation wurde noch nicht als Diplomarbeit oder ähnliche Prüfungsarbeit verwendet.

Hannover, 28.03.2003

Annely Haase

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All the experiments, practical work, and documentation were done by Annely Haase. The manuscript were written by Annely Haase under the supervision of Prof. Dr. G. Bicker, who helped to interpret and discuss the results of the work.

Abbreviations

Abbreviation	Full name
AC	adenylyl cyclase
cAMP	adenosine 3´,5`-cyclic monophosphate
cGC	soluble guanylyl cyclase
cGMP	guanosine 3´,5`-cyclic monophosphate
NADPHd	nicotinamide adenine dinucleotide phosphate diaphorase
7NI	7-nitroindazole
NO	nitric oxide
NOS	nitric oxide synthase
ODQ	1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one
PBS	phosphate buffered saline
PKA	protein kinase A
PKG	protein kinase G
RPcAMPS	8-Bromo adenosine 3`, 5`-cyclic monophosphothioate, RP-Isomer
RPcGMPS	8-Bromo-guanosine 3`, 5`-cyclic monohosphothioate, RP-Isomer
s.e.m.	standard error of median
SNP	sodium nitroprusside
SPcAMPS	Adenosine 3´.5´-cyclic monophosphothioate, SP-Isomer,

Contents

1. Introduction

Cell migration plays an important role in a wide variety of biological phenomena. In embryogenesis, cellular migration is a recurring theme in morphogenic processes ranging from gastrulation to the development of nervous systems. Migration remains prominent in the adult organism, in normal physiology as well as in pathology. In the inflammatory response for example, leukocytes immigrate into areas of insult, where they mediate immune and phagocytic functions. Migration of vascular endothelial cells and fibroblasts is essential for wound healing. Cell migration gone awry can lead to birth defects or tumour metastasis. In metastasis, tumour cells migrate from the initial tumour into the circulatory system, which they subsequently leave to migrate into new sites. Finally, a better understanding of cell migration is crucial to technological applications such as tissue engineering, playing an essential role in colonization of biomaterials scaffolding (reviewed in Lauffenburger and Horwitz, 1996).

1.1 Neuronal migration

Neuronal migration is central to the development of nervous systems. Neurons are usually born in specialized proliferative zones but finally reside in distinct locations of the mature nervous system. Thus, directed cell migration is necessary to bring the neurons to their ultimate destination (Hatten, 1999). In the mammalian central nervous system, for example, virtually all neurons migrate from their site of origin, near the cerebral ventricles, to distant regions where they establish permanent residence. Once the cells have finished dividing, they dislodge from adjacent progenitors, extend leading processes and then move along specific migratory pathways. The directed migration of neurons along specific pathways is essential to the formation of both the central and peripheral nervous system. Particularly during the development of the peripheral nervous system, neurons migrate over long distances to their final locations in the periphery. Neural crest cells, for example, emerge from the dorsal margin of the neural tube and migrate over long distances along specific routes to form sensory, autonomic and enteric ganglia in the peripheral nervous system (Le Douarin et al., 1994). After completing migration at their final destination, each neuron starts to extend an axon and dendrites and develops specific synaptic contacts characteristic to its phenotype (Hatten, 1999).

The current view of cell migration is that motile cells extend and retract numerous actin-rich protrusions into the surrounding followed by a translocation of the cell body (reviewed by Lauffenburger and Horwitz, 1996). In particular, it begins with a spatial asymmetry within the cell, with polarized morphology which manifest in a clear distinction between the cell front and cell rear. To migrate, cells form extensions of the plasma membrane, including both lamellipodia and filopodia, primarily at the leading edge of the cell. Lamellipodia are flat, broad, sheet-like structures, whereas filopodia are thin, cylindrical, needle-like projections. Cytoplasmic organelles are excluded from these structures, which abundantly contain actin and actin-associated proteins (Lauffenburger and Horwitz, 1996). These protrusions are driven by the polymerization of a network of actin filaments and stabilized through the formation of adhesive complexes. If sufficiently strong adhesion develops between the membrane extension and the substratum, then retraction is prevented, stabilizing certain protrusions while less-adherent extensions disappear. The adhesive complexes are regions of the plasma membrane where integrin receptors, actin filaments, and associated proteins cluster together. As the cells migrate, the small nascent adhesive complex (focal complex) at the front of the cell grow and strengthen into larger, more organized adhesive complexes (focal adhesions) that serve as points of traction over which the body of the cell moves. Finally, release of adhesion at the rear of the cell results in a displacement of the cell (Lauffenburger and Horwitz, 1996).

1.2. The actin cytoskeleton

Neuronal cell migration requires the dynamic regulation of the actin cytoskeleton (Ballestrem et al., 1998). Actin is a highly conserved and abundant cytoskeletal protein in all eukaryotic cells. It is implicated in a number of cellular activities, including mitosis, cell shape-changes and motility (Lauffenburger and Horwitz, 1996; Ballestrem et al., 1998). Many of these processes require the dynamic regulation of the actin cytoskeleton. These dynamic structural alterations require polymerization, depolymerization, stabilization, branching, and severing of actin filaments (Ballestrem et al., 1998). Actin is involved in a wide range of structures, from stiff and relatively permanent extensions of the cell surface to the dynamic three-dimensional network at the leading edge of the cell. Very different structures based on actin coexist in the cell. However, in every case, the fundamental structure of actin is the same. It is the length of these filaments, their stability and For migration, the cell cortex is one of the most important parts of the cell. This

actinrich layer beneath the plasma membrane gives the cell the mechanical strength and enables it to perform a variety of cell surface movements, such as phagocytosis, cytokinesis, and cell locomotion. In the cell cortex, actin filaments are organized into three general types of arrays. In parallel bundles, as found in filopodia, the filaments are orientated with the same polarity and are often closely spaced (10 – 20 nm apart). In contractile bundles, as found in stress fibres of fibroblasts for example, or in the contractile ring that divides the cells during mitosis, filaments are arranged with opposite polarities and contain the motor protein myosin-II; they are more loosely spaced (30 - 60 nm). The third group of filaments are the gel-like networks. The filaments are arranged in a relatively loose, open array with many orthogonal interconnections. These different arrangements of actin filaments are generated and maintained by actin filament cross-linking proteins. For example, Fimbrin is enriched in the parallel bundles of the leading edge, particularly in filopodia and is thought to be essential for the tight binding of actin filaments in these structures. Another actin filament cross-linking proteins, α-actinin is responsible for the cross linking of contractile bundles and for the anchorage of fibre bundles in focal adhesions (Alberts et al., 1994; Zigmond, 1996).

Attachments between the actin filaments inside the cell and the extracellular matrix on the outside of the cell are mediated by transmembrane glycoproteins. The main transmembrane glycoproteins of focal adhesions are members of the integrin superfamily, whose external domain binds to an extracellular matrix component while the cytoplasmic domain is linked to contractile actin fibre bundles. The linkage is indirect and mediated by multiple attachment proteins. The current view of actin anchorage is that the cytoplasmic domain of integrin proteins binds to the protein talin, which in turn binds to vinculin. Vinculin associates with α-actinin and is thereby linked to an actin filament. Besides their role as anchors for the cell, focal adhesions can also relay signals from the extracellular environment to the cytoskeleton. Several protein kinases, including tyrosine kinases of the src family or focal adhesion kinases, are localized to focal contacts or focal adhesions. Their activity can change with the type of substratum and once activated, these kinases can phosphorylate various target proteins, including components of the cytoskeleton (Alberts et al., 1994; Zigmond, 1996).

In stationary cells, the actin fibres form a dense network of actin fibres that fix the cells to the substratum via focal adhesions complexes. During migration, the actin cytoskeleton undergoes a fundamental reorganization. The dense net of actin bundles dissociates and new actin filaments are formed at the leading edge of the

cell, including filopodia and lamelipodia (Brown et al, 1999). Rapid extension and retraction of filopodia and lamellipodia through F-actin assembly and disassembly are the driving forces that permit cells to explore and navigate in response to extracellular cues (Woodring et al., 2002). While it seems that the regulated activity of cell adhesion molecules, actin cytoskeleton, and its associated proteins are essential to cell migration, the complete molecular mechanisms that control cell migration are yet unknown (Montell, 1999; Horwitz and Parsons, 1999).

1.3. Interactions between the migrating neuron and the environment

During migration, interactions between the neurons and the neighbouring cells and the surrounding environment are essential for the regulation of the migratory behaviour. Malfunctions in cellular communication could result in perturbation of migration. During embryogenesis, defects of neuronal migration lead to severe malformations of the brain. For example, classic lissencephaly is a severe human brain malformation characterized by an absence or reduction of the normal cerebral convolutions. In the lissencephalic brain, the cerebral cortex is abnormally thickened and lacks its laminar structure and the normal morphology of cortical neurons is disrupted. Several lissencephaly mutations in human and mouse genes such as *Reelin*, *Disabled*, or *LIS1n* have been found to cause defects in cortical neuronal migration. *Reelin* for example, an extracellular matrix protein secreted by the earliest-born neurons in the brain, serves as a 'stop' signal for migrating neurons and regulates integrin-mediated adhesion between neurons and radial glial cells (Dulabon et al., 2000; reviewed in Feng and Walsh, 2001). Thus, the interactions between the migrating neurons and the neighbouring cells are essential for the selection of the right pathways, as well as for orientating, conducting and stopping neuronal movement at the appropriate site (Rakic, 1999).

Migration is regulated by extracellular guidance factors such as cell surface, extracellular matrix, and diffusible signalling molecules which have to be interpreted by the intracellular signal transduction machinery that drives the cytoskeletal rearrangements leading to directed movement of the neuron (Song and Poo, 2001). The guidance of both the migrating nerve cell and its growth cones depends on positional cues in the environment of the embryonic nervous system. An emerging theme in neuronal navigation is that axon guidance cues can also guide directed migration of nerve cell bodies (Wu et al. 1999; Zhu et al. 1999; Song and Poo, 2001; Causeret et al., 2002). The secreted protein Slit, for example, is a

chemorepellent for commissural axons in *Drosophila*. (Battye et al., 1999; Kidd et al., 1999). However, Slit also acts as a repellent for the migration of interneurons from the subventricular zone of the telencephalon to the olfactory bulb (Wu et al.1999) and the migration of GABAergic neurons from an extracortical origin to the embryonic neocortex (Zhu et al., 1999), and the correct ventral positioning of inferior olivary neurons (Causeret et al., 2002). Ephrins serve as guidance signals for the projection of retinal axons in the optic tectum and for the migration of trunk neural crest cells across the somites (Krull et al., 1997; Loschinger et al., 2000). In these examples, the same set of membrane receptors are involved in both axon guidance and neuroblast migration, suggesting that cellular signal transduction mechanisms for these two forms of navigation may be similar.

1.4. Nitric oxide signalling

An increasing number of investigations have implicated the unconventional signalling molecule nitric oxide (NO) and its main effector, the soluble guanylyl cyclase enzyme in mechanisms of neurite growth. NO is a short-lived messenger that diffuses from its site of production and is thought to move readily through cell membranes. NO is generated by Ca^{2+}/calmodulin-dependent nitric oxide synthases (NOS). NOS catalyze the production of NO and L-citrulline from L-arginine, oxigen, and NADPH-derived electrons. The principle function of NO appears to be as an activator of the heterodimeric heme protein soluble guanylyl cyclase (sGC), although other signal transduction mechanisms are possible (reviewed in Bredt and Snyder, 1992; Dawson and Snyder, 1994; Garthwaite and Boulton, 1995). Binding of nanomolar concentrations of NO to the prosthetic heme group induces sGC to catalyze the formation of cyclic GMP (cGMP). The specifity of cellular communication is preserved by the activity dependent release of NO and discrete distribution of the target receptor enzyme sGC. Synthesis of cGMP may directly gate ion channels, stimulate protein kinase G (PKG) and cGMP dependent phosphodiesterases, and regulate additional other downstream signal transduction cascades (Wang and Robinson, 1997).

The dynamic regulation of NOS and sGC during the formation and regeneration of the nervous system (Bredt and Snyder, 1994; Roskams et al., 1994; Brüning and Mayer, 1996) has lead to the suggestion that NO/cGMP signalling functions in developmental processes. Evidence from developmental studies in both vertebrates and invertebrates suggests an involvement of NO/cGMP signalling in neurite

growth, synaptogenesis, and synaptic maturation processes (Wu et al., 1994; Wang et al., 1995; Cramer et al., 1996; Truman et al., 1996; Ball and Truman, 1998; Gibbs and Truman, 1998; Wildemann and Bicker, 1999a, Seidel and Bicker, 2000; Leamey et al., 2001, Seidel and Bicker, 2002). Notably, the developmental expression of these enzymes in many neurons is transient, coinciding with particular stages of embryogenesis and often overlapping with periods of axonal outgrowth and synaptogenesis. Investigations on cultured neurons have reported that NO affects a multitude of growth cone behaviours (Hess, et al., 1993; Renteria and Constantine-Paton, 1995, He et al., 2002; Hindley et al., 1997; Poluha et al., 1997; Van Wagenen and Rehder, 1999). For example, experimental manipulation of NO signal transduction in the vertebrate nervous system indicate that NO mediates the refinement of retinotectal projections (Wu et al., 1994; Cramer et al., 1996). At the developing neuromuscular synapses, manipulations of the NO signalling system lead to activity-dependent synaptic suppression (Wang et al., 1995). Moreover, the recent analysis of cGMP-dependent proteinkinase I deficient mice has revealed axon guidance defects of sensory neurons (Schmidt et al., 2002).

NO signalling have been detected in a variety of invertebrates, indicating that this signalling pathway is evolutionary conserved. Neurochemical investigations have shown the presence of Ca^{2+}/calmodulin-stimulated NOS activity and NO-induced sGC activity in invertebrate nervous systems such as in the fruit fly (*Drosophila melanogaster*), in grasshoppers (*Locusta migratoria*, *Schistocerca gregaria*), in the honey bee (*Apis mellifera*) and the tobacco hornworm (*Manduca sexta*). Isoforms of both NOS and sGC have been isolated and characterized in the insect nervous system. (liu et al. 1995; Simpson et al. 1997; Nighorn et al., 1998). Demonstrating, that the cellular mechanisms of NO/cGMP signalling are not peculiar to the mammalian brain but apply to insects as well (reviewed in Bicker, 2001).

In the embryonic grasshopper, for example, synaptogenesis correlates with a phase when many identifiable nerve cell types respond to NO by producing cGMP (Truman et al., 1996; Ball and Trumann, 1998). Inhibition of NOS and sGC results in a reduction of terminal synaptic branch formation in a migratory population of embryonic *Manduca* neurons (Wright et al., 1998). During visual system formation in *Drosophila* pupae, the photoreceptors respond to NO stimulation with the synthesis of cGMP during a specific temporal window, while the postsynaptic optic ganglia stain for NADPH-diaphorase, a histochemical marker for NOS (Gibbs and Truman, 1998). Pharmacological manipulation of NO/cGMP signal transduction *in vitro* during the period of NO sensitivity disrupts the establishment of proper retinal

connections in the optic lobes of drosophila (Gibbs and Truman, 1998). In *Drosophila*, the formation of neuromuscular synapses begins in the embryo but synapse maturation continues during the entire larval development, when muscle fibres increase in size (Keshishian et al., 1996; Davies and Goodman, 1998; Prokop, 1999). There are certain evidences for a retrograde signalling between motor neuron and muscle mediated by NO/cGMP signalling (Wildeman and Bicker, 1999).

1.5. Cyclic nucleotide signalling

Considerable progress has also been made in understanding how extracellular guidance cues are transduced to regulate the pathfinding of a neurite (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Song and Poo, 2001; Yu and Bargman, 2001; Dickson, 2002). In the process of neuronal navigation, extracellular signals like NO have to be interpreted by the intracellular signal transduction machinery that drives the cytoskeletal rearrangements leading to fibre outgrowth or directed migration. Using an *in vitro* approach, it has been demonstrated that the response to an extracellular guidance cue can be converted from attraction to repulsion, by changing the intracellular levels of cyclic nucleotides. For example, the response of dissociated *Xenopus* spinal neurons to netrin-1 could be converted between repulsion and attraction by altering the cyclic AMP (cAMP) level in the growth cone (Ming et al., 1997). Elevated levels of cGMP can also change the response of growth cones to a semaphorin from repulsion to attraction (Song et al., 1998). Remarkably, an asymmetric cellular localization of the cGMP synthesising enzyme soluble guanylyl cyclase (sGC) to the dendrite of pyramidal cells is thought to confer the opposite directional outgrowth to dendrites and axons in a semaphorin gradient of the cerebral cortex (Polleux et al., 2000). Thus, intracellular cyclic nucleotide levels can be the critical factors that govern process extension to the same chemotropic guidance cue.

Moreover, cyclic nucleotides have been shown to modulate growth cone behaviour *in vivo*. Qiu et al. (2002) could demonstrate that elevation of cytosolic cAMP in lesioned spinal axons of rats is sufficient to overcome the inhibitory effect of myelin-associated glycoprotein, a component of myelin and a potent inhibitor of axonal regeneration. In consequence, the elevated cAMP levels induced regeneration in these lesioned neurons. Recent analysis of cGMP-dependent proteinkinase I deficient mice has revealed axon guidance defects of sensory neurons (Schmidt et

al., 2002). In the grasshopper nervous system, cGMP is a permissive regulator of pioneer axon extension. Similar to the pioneer neurons in the embryonic legs, the first neural pathway in the antennae of the grasshopper are established by identified pairs of pioneer neurons (Bate 1976; Ho and Goodmann 1982). These pioneer neurons synthesize cGMP in response to exogenous NO treatment. Pharmacological inhibition of endogenous guanylyl cyclase and of NO synthase results in an abnormal pattern of pathway formation in the antenna. The pharmacological perturbation of pioneer axon pathfinding can be rescued by supplementing with membrane permeant cGMP (Seidel and Bicker; 2000). Thus, cyclic nucleotides such as cGMP and cAMP are central components in the intracellular signalling machinery conducting growth cone behaviour during the development of nervous systems. However, a possible role of NO/cyclic nucleotide signalling in the process of neuronal migration remains to be elucidated.

The migration of neurons seems to involve many of the same aspects of cellular regulation that characterize the migration of non-neuronal cells (Montell, 1999; Horwitz and Parsons, 1999). In non-neuronal cells, the influence of cyclic nucleotide signalling on cell migration is well studied. Migration-stimulatory effects of cGMP are found on macrophages, endothelial cells and epithelial cells (Elferink and Van Uffelen, 1996; Ziche et al., 1994; Noiri et al., 1996). In primary cultured smooth muscle cells, the migration stimulatory effect of NO donors and cGMP is associated with altered cell morphology and dissociation of actin filaments (Brown et al, 1999), whereas, in the same cell type, cAMP donors and activators of PKA exert an inhibitory effect on migration (Sun et al., 2002). Compelling evidence for the involvement of cyclic nucleotide signalling in the process of cell migration come from a cAMP mutant mouse. Increased basal activity of the cAMP effector enzyme PKA in a mutant mouse model leads to reduced ability of mesodermal cell migration. In primary cultured fibroblasts from these mutant mice, the cells displayed reduced ability of migration and an abnormal cytoskeleton with disorganized actin bundles whereas the wildtype cells only displayed sparse parallel actin fibres (Amieux et al., 2002).

To this end, a role of NO/cyclic nucleotide signalling in mechanisms of neuronal migration during development has not been uncovered.

1.6. The insect nervous system as model system for neuronal migration

This thesis attempts to provide new insight into guidance molecules and intracellular signal transduction pathways that influence neuronal migration during the development of the enteric nervous system of the grasshopper embryo. To investigate a potential function of NO/cyclic nucleotide signalling during the processes of neuronal cell migration it would be helpful to find systems where NO signalling can be analyzed in the intact embryo. Thus, insects with their relatively simple nervous system and accessibility to pharmacological and genetic manipulations are attractive preparations for the analysis of NO/cyclic nucleotide signalling during development (reviewed in Bicker, 2001a). To examine a potential function of NO/cGMP signalling and cAMP/protein kinase A signalling during neuronal migration I have chosen the grasshopper (*Locusta migratoria*, L.) as a model system. The grasshopper embryo is a well-established model with which to study the cellular and molecular mechanisms during neuronal development (Goodman et al., 1984; Bentley and O'Connor, 1992). In addition, the grasshopper provides an excellent histochemical approach to the components of the NO/cGMP signal pathway. Moreover, using an embryo culturing system, the grasshopper is accessible to pharmacological manipulations throughout embryogenesis (Seidel and Bicker, 2000).

To study neuronal cell migration, it is important to use a system where migration can be analyzed in the intact embryo. Generally, many studies of cell migration are carried out on cultured cells because the movement can be observed in real time and the culture medium can be altered to examine the factors that affect cell motility. However, during embryonic development, cell migration must be regulated temporally and spatially and this behaviour must be coordinated with cell fate specification and differentiation of the motile cell. Thus, *in vitro* studies can suggest, but cannot establish, the nature of the factors that act to control cell migration or guide migrating cells *in vivo* (Montell, 1999). Therefore, it is important to have an experimental system like the grasshopper embryo, in which cell migration can be studied *in vivo*.

1.7. Development of the enteric midgut plexus

While the phenomenon of neuronal migration was first characterized in vertebrates, it is now clear that this same process is also critical to the formation of invertebrate nervous systems. The formation of the insect enteric nervous system (ENS) (reviewed in Hartenstein, 1997) provides a well established model to study the cell biology of neuronal migration. During the development of the hawk moth *Manduca*, cell migration of enteric plexus (EP) neurons requires the cell adhesion molecule fasciclin II, and Ca^{2+}-mediated cellular effects of G proteins (Horgan and Copenhaver, 1998; Wright et al., 1999; Wright and Copenhaver, 2000). Since migrating EP cells began to synthesize cGMP in response to the application of exogenous NO, pharmacological blockers of NO/cGMP signal transduction have been applied during embryonic development. However, a role of NO-sensitive guanylyl cyclase signalling has only been uncovered in post migratory synaptic branch formation, but not during the actual process of cell migration (Wright et al., 1998).

In the present study, I examine the role of NO/cGMP/PKG and cAMP/PKA signalling pathways in the regulation of neuronal migration in the ENS of the grasshopper (*Locusta migratoria*, L.). I focus on the directed migration of one set of enteric neurons, the midgut plexus neurons (MG neurons), which ultimately populate a nerve plexus that spans the midgut (Ganfornina et al., 1996).

The enteric nervous system of the grasshopper is organized into four interconnected ganglia located on the foregut and two separate plexuses that innervate the foregut and the midgut. The foregut plexus is composed of neurons dispersed over the surface of the stomodeum, whereas the midgut plexus is arranged in four main longitudinal pathways running along the midgut surface (Ganfornina et al., 1996). During the formation of the midgut plexus, postmitotic neurons arise in a neurogenic zone on the foregut and then migrate laterally to accumulate at the posterior edge of the ingluvial ganglia. At 60% E, these midgut neurons (MG neurons) migrate posterior and assemble on the foregut-midgut boundary, organized into four cellular packets (Fig. 2). The leading MG neurons bear neurites that are connected to the ingluvial ganglia (Ganfornina et al., 1996). At 62% of development, the MG neurons are engaged in a phase of rapid migration, during which the neurons migrate up to 300 µm in 24 hours. Subsets of MG neurons

detach from the cell packets and migrate posterior along the midgut surface. The migrating neurons are of bipolar morphology with short processes running posteriorly. The processes that are trailing behind establish the four midgut nerves, two derived from each ingluvial ganglion (Ganfornina et al., 1996). At 70% E, migrating MG neurons can be seen along the entire length of the midgut nerves. The leading MG neurons travel approximately 85% of the total length of the midgut until they complete migration. Between 70% and 75% of development, the first lateral branches appear (Fig. 2). At 80% E, some MG neurons leave the main migratory routes to spread out between the midgut nerves. These post-migratory neurons extend terminal synaptic branches on the midgut musculature. At 95% E the phase of lateral migration and axonal branching is completed and the midgut plexus shows a mature organization. The migrating neurons are presumably peripheral sensory neurons, whereas the processes consist of both afferent fibres from sensory neurons and efferent motor fibres from cells located in the enteric ganglia (Ganfornina et al., 1998).

This thesis provides evidence that NO and cyclic nucleotides influence the migration of the midgut neurons. The MG neurons exhibit inducible cGMP-immunoreactivity (cGMP-IR) throughout the phase of migration and continue to show high levels of anti-cGMP staining in the phase of lateral axon branching and the formation of terminal processes on the midgut musculature. When the midgut plexus acquires its mature configuration, the cGMP-IR decreases. Using NADPH-diaphorase staining as a histochemical marker for NOS, I identified potential sources of NO in subsets of the midgut cells below the migrating MG neurons.

To examine a functional role of the NO/cGMP and the cAMP/PKA pathway during the development of the midgut plexus, I cultured whole grasshopper embryos for 24 hours in neurochemicals known to affect these signalling pathways. Pharmacological inhibition of endogenous NOS, sGC, and PKG activity in embryo culture results in a significant retardation of MG neuron migration. This pharmacological perturbation of MG neuron migration can be rescued by supplementing with membrane-permeant cGMP and protoporphyrin IX free acid, an activator of sGC.

Moreover, I could demonstrate that NO/cGMP and cAMP/PKA signalling have antagonistic effects on MG neuron migration. Using the embryo culture system, I show that pharmacological elevation of cytosolic cAMP and direct stimulation of

PKA results in an inhibition of normal migration, whereas, the PKA inhibitor RPcAMPS had no disruptive effect on MG neuron migration.

Furthermore, I elucidate the effects of cAMP and cGMP on the actin cytoskeleton of the migratory neurons. To examine the dynamic regulation of the F-actin cytoskeleton in MG neurons under the influence of different neurological pharmaca known to affect the cGMP/PKG or the cAMP/PKA signaling pathways, I used a combined embryo culture/cell culture system. In conclusion, under conditions where migration proceeds, I observed a disassembly of actin fibres in the migrating MG neurons. In these cells, F-actin staining was exclusively found in the neurites and the cell cortex, whereas in the cell body, F-actin staining was absent. In contrast, under conditions where MG neuron migration was disturbed, the cells showed a non-migratory phenotype in that prominent F-actin fibers formed a dense net of actin-bundles in the soma.

Thus, this investigation reveals an accessible *in vivo* system in which the role of NO and cyclic nucleotide signalling during neuronal migration can be analyzed at a cellular level.

2. Material and Methods

Locusta migratoria eggs were collected from a crowded laboratory culture and kept in moist petri dishes at 30°C. Staging of the embryos was based on the system of Bentley et al. (1979) with additional criteria for later embryos (Ball and Truman, 1998). An optimal stage for *in vivo* culturing and pharmacological manipulation was indicated by the first appearance of brownish pigmentation at the tips of the antennae.

2.1. Visualization of sGC and NOS activity

Anti-cGMP immunocytochemistry

Embryos of stages 55% - 95% E were opened dorsally and guts were dissected out in ice cold L15 medium. To induce activity of sGC, guts were exposed to SNP (100 µM) in the presence of the phosphodiesterase inhibitor IBMX (1 mM) for 20 minutes at room temperature (De Vente et al., 1987; Seidel and Bicker, 2000). The guts were fixed in Pipes-FA overnight at 4°C and permeabilised in 0.3% Saponin-PBS for 1 hour. After rinsing in PBT and blocking in PBT/5% normal rabbit serum for 2 hours, the primary sheep anti-cGMP antiserum (courtesy of Dr. Jan De Vente; see Tanaka et al., 1997) was applied at a dilution of 1:5000 in blocking solution at 4°C overnight. Subsequently, the guts were exposed to a biotinylated rabbit anti-sheep antibody (Vector, diluted 1:250). Immunoreactivity was visualised by standard peroxidase staining techniques using the Vector ABC kit. After incubating in ABC-solution, the guts were preincubated in 70% ethanol for 5 – 10 minutes, washed several times in PBS and subsequently stained with a DAB-kit. In the absence of SNP stimulation, cGMP-IR could not be detected by this immunocytochemical method.

To elucidate whether cGMP expression of MG neurons is developmentally regulated during embryogenesis, anti-cGMP stained guts were counterstained with anti-acetylated α-Tubulin which stains all MG neurons, as described above. For the stages 55% to 95% E, the total number of α-Tubulin and cGMP-positive MG neurons was calculated for each preparation. The mean values and s.e.m. were calculated for each developmental stage (N = 5).

NADPH-diaphorase staining

For NADPH-diaphorase histochemistry, guts of stages between 50% and 95% were dissected in L15 medium. The guts were fixed in 4% Pipes-FA with 10% methanol for 1 hour on ice. Subsequently, the preparations were permeablised in 0.3% Saponin-PBS for 30 minutes. After rinsing in 50 mM Tris-HCL (pH 7.8), the guts were incubated in 0.1 mM ß-NADPH/0.1 mM Nitro Blue Tetrazolium in Tris-HCL at room temperature (in the dark) for 1 hour. Subsequently, the tissue was repeatedly washed in PBS and cleared in glycerol series. To control for specificity of the histochemical staining, NADPH was omitted from the reaction, which resulted in no detectable staining of the cells.

2.2. *In vivo* culturing experiments

Embryo culture

Embryos used for culture experiments were carefully staged between 60% and 63% of embryonic development (% E). During these stages, the MG neurons had not migrated more than 40 µm along the midgut surface. Embryos of one clutch were randomly divided into groups that were exposed to the pharmacological substances. Eggs were sterilized in 70% ethanol and dissected in sterile cell culture medium (L15, Gibco, Life Technologies), supplemented with 1% penicillin-streptomycin solution (10,000 units/ml). After removal from their egg cases, the embryos were restrained in Sylgard chambers and covered with sterile cell culture medium. A small incision in the dorsal epidermis was created immediately above the foregut. Embryos were then allowed to develop for 24 hours at 30°C. The incision did not reseal and thus, the developing ENS was exposed to the pharmacological agents during the whole culturing period. Finally, guts were dissected out of the embryos and fixed in Pipes-FA (100 mM Pipes, 2.0 mM EGTA, 1 mM $MgSO_4$, 4% paraformaldehyde, pH 7,4) overnight at 4°C.

Neuropharmaca

To test for developmental functions of the NO/cGMP and cAMP/PKA signalling, I used neurochemicals that are known as inhibitors or activators of these signal transduction systems (Fig. 1). The NOS inhibitor 7-nitroindazole (7NI), the sGC activator protoporphyrin IX free acid (Alexis), and the sGC inhibitor 1H-[1,2,4]-

oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, Alexis, San Diego, CA) were dissolved in dimethyl sulfoxide (DMSO) to provide a final concentration of 5 mM DMSO in culture medium. Control cultures contained the same concentration of DMSO. The NO-donor sodium nitroprusside (SNP, Sigma), the phosphodiesterase inhibitor 3-isobutyl-methylxanthine (IBMX, Sigma), the cGMP analogue 8-Bromo-cGMP (8Br-cGMP, Sigma) and the PKG inhibitor 8-Bromo-guanosine 3´,5´-cyclic monohosphothioate RP-Isomer (RPcGMPS, Alexis) were dissolved directly in L15 medium. The cAMP donor Forskolin (Alexis), the PKA activator Adenosine 3´.5´-cyclic monophosphothioate, SP-Isomer, (SPcAMPS, Alexis), the PKA inhibitor 8-Bromo adenosine 3`, 5`-cyclic monophosphothioate RP-isomer (RPcAMPS, Alexis), and the cAMP analogue 8-bromo-cAMP (8Br-cAMP, Sigma) were dissolved in L15 medium.

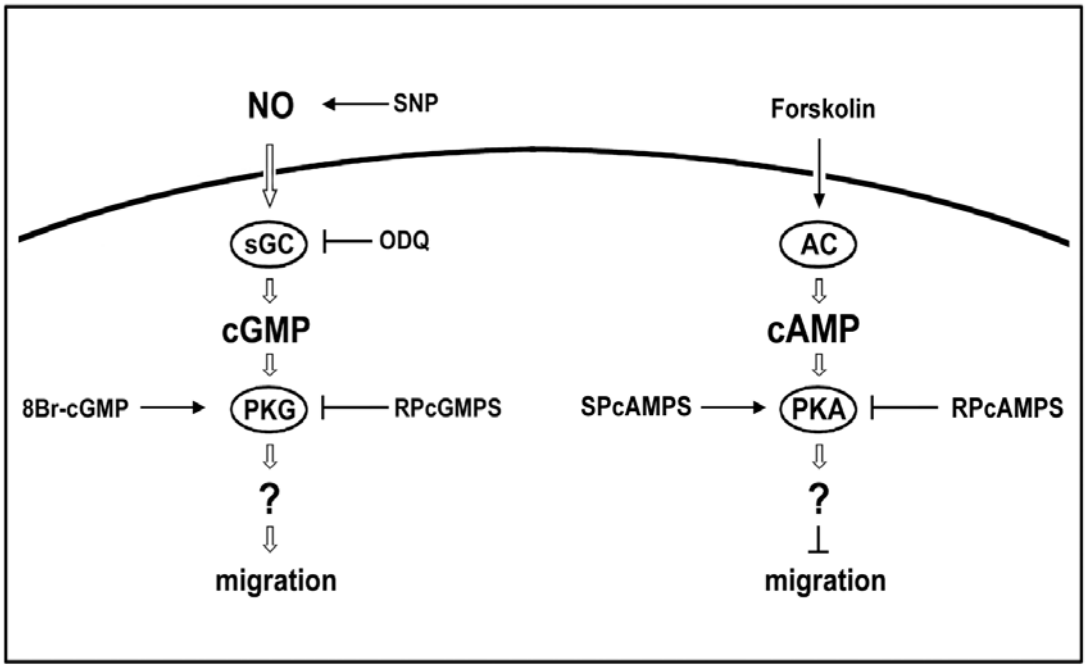

Fig. 1 Action of neurochemicals affecting the NO/cGMP and cAMP/PKG signal transduction

Fig. 1: As enzyme inhibitors of the NO/cGMP pathway, The specific NO-synthase (NOS) inhibitor 7-nitroindazole (7NI), the soluble guanylyl cyclase (sGC) inhibitor 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-1-one (ODQ), and the protein kinase G (PKG) inhibitor 8-Bromo-guanosine 3´,5´-cyclic monohosphothioate, RP-Isomer (RPcGMPS) were applied. To stimulate sGC activity, the NO-donor sodium nitroprusside (SNP) and the sGC activator protoporphyrin IX free acid, which stimulates sGC independently of NO were used. The membrane permeable 8Br-cGMP was used to raise cGMP levels directly. As enzyme activators of the cAMP/PKA pathway, Forskolin, a specific stimulator of the enzyme adenyly cyclase and Adenosine 3´.5´-cyclic monophosphothioate, SP-Isomer, (SPcAMPS) as a specific activator of protein kinase A (PKA) were applied. As inhibitor of PKA, 8-Bromo adenosine 3`, 5`-cyclic monophosphothioate RP-isomer (RPcAMPS) was used.

Anti-acetylated α-Tubulin immunocytochemistry

To visualize the migrating neurons on the midgut, the preparations were labelled with an antiserum against acetylated α-tubulin (Sigma), a characteristic feature of stable microtubular arrays in axons. This antiserum stains somata and neurites of the MG neurons (Ganfornina et al., 1996).

The fixed guts were permeablised in 0.3% Saponin-PBS (phosphate-buffered saline, pH 7.4) for 1 hour. Subsequently, guts were blocked in 5% normal goat serum in PBT (PBS + 0,3% BSA + 0,5% Triton) for 1 hour. The polyclonal mouse antiserum against acetylated α-tubulin was applied in a dilution of 1:250 in PBT/5% normal horse serum at 4°C overnight. After washing several times in PBT, the guts were exposed to a biotinylated horse anti-mouse antibody (Vector, diluted 1:250). For visualization of anti α-tubulin immunoreactivity, I used Streptavidin-Cy3 (Sigma, diluted 1:250) as fluorescent marker. Guts were cleared in a glycerol series (50%, 90% in PBS) and mounted in Vectashield (Vector).

Analysis of MG neuron migration

Immunocytochemically stained preparations were observed using a Zeiss Axiovert microscope. Pictures were captured with an AxioCam HRc linked to Zeiss image processing system. To quantify the cell migration on the midgut surface, I measured the distance from the foregut-midgut boundary to the position of the leading MG neuron (Wright et al., 1998). I employed a Mann-Whitney U-test for statistical comparisons of the means of the experimental and the control groups. Histogram values indicate the mean values ± s.e.m. as percentage of the matched control values of each experiment. $N \geq 20$ embryos for each histogram. All significance levels are two-tailed. For visualisation of the migration pattern on the midgut, captured images of the immunolabelled MG neurons were traced and arranged as drawings.

2.3. Primary cell culture of MG neurons

To establish the primary cell culture, in a first step, the embryos were precultured with the pharmacolocical substances for 24 hours as described above (see embryo culture). In a second step, the guts were dissected out and the MG neurons were immediately blotted on a coated glass coverslip.

In detail, after the incubation period, the gut was dissected out. Subsequently, the yolk was removed by forceps and the gut was transferred to a petri dish containing 3 ml L15 medium and a poly-L-Lysine coated coverslip at the bottom. Under the binocular, the gut was carefully rolled on the coverslip. The MG neurons stacked at the coated surface, whereas the gut epithelium kept intact during the blotting procedure and was finally removed from the petri dish. The blotted cells immediately were fixed with Pipes-FA for 15 - 30 min on ice.

Phalloidin/anti-HRP co-Staining

To visualize the actin cytoskeleton in MG neurons, F-actin was stained with AlexaFluor 568 phalloidin (Molecular Probes). To verify the neuronal identity of the blotted cells, I used a primary antiserum against horseradish peroxidase (anti-HRP, Jackson Immunoresearch) which is known to stain a carbohydrate epitope, selectively expressed by membrane proteins on the surface of insect neurons (Jan and Jan, 1984).

After rinsing several times with PBS, the blotted neurons were fixed in 4% Paraformaldehyde/PBS on ice for 15 minutes, subsequently permeablised in PTX 0,5% for 1 min and frequently washed in PBS. The MG neurons were blocked in 5% normal horse serum/PBT for 15 min. The primary goat anti-HRP antiserum was applied in a dilution 1:5000 in blocking solution for 30 min. Subsequently, the neurons were rinsed in PBS and exposed to a biotinylated secondary antibody (Vector, diluted 1:250). Immunoreactivity was visualized using Streptavidin Alexa Fluor 488 conjugate (Molecular Probes). For F-actin staining, the cells were incubated in Alexa Fluor 568 Phalloidin (6.6 µmol stock solution in 200 µl PBS).

I found a difference between embryos treated with pharmacological reagents and control cultured embryos with respect to the F-actin distribution in the MG neurons. In control experiments, MG neurons showed a phenotype with Phalloidin staining

mainly in the neurites and the cell cortex (termed migratory phenotype). Whereas in MG neurons treated with NO/cGMP pathway inhibitors or cAMP/PKA pathway activators, F-actin staining was mainly found in the cell somata (termed non-migratory phenotype). To quantify these results, I examined 50 neurons in each cell culture (= 1 gut) and counted the number of cells showing a migratory phenotype or a non-migratory-phenotype respectively (N = 5 guts for each experiment). A Mann-Whitney U-test was employed for statistical comparisons of the means of the experimental and the control groups. Histogram values indicate the mean values ± s.e.m. as percentage of the matched control values of each experiment. N = 5 embryos for each histogram. All significance levels are two-tailed.

Anti-cGMP immunohistochemistry

To determine whether NO sensitive sGC activity is present in cultured MG neurons, I dissected the guts of untreated embryos (60% to 65% E) and blotted the MG neurons on coverslides as described above. The cells were kept in culture for 24 hours or 28 hours at 30° C. To induce sGC activity in the MG neurons, the blotted neurons were subsequently exposed to SNP (100 µmol) in the presence of IBMX (500 µmol) for 20 minutes at room temperature (De Vente et al., 1987; Seidel and Bicker, 2000). The cells were fixed in Pipes-FA for 30 min at room temperature and permeablised in 0.3% Saponin-PBS for 5 min. After rinsing in PBT and blocking in PBT/5% normal rabbit serum for 30 min, the primary sheep anti-cGMP antiserum was applied at a dilution of 1:5000 in blocking solution for 1 hour. Subsequently, the neurons were exposed to a biotinylated rabbit anti-sheep antibody (Vector, diluted 1:250). Immunoreactivity was visualised using Streptavidin-Cy3 (Vector) as fluorescence marker.

2.4. Time-lapse video microscopy

For *in situ* video microscopy of living MG neurons I used embryos staged between 64% and 68% E. During this stage, the MG neurons are still migrating on the midgut surface, but the living gut tissue is stable enough to endure the staining procedure. At about 68% E, the initiation of first peristaltic movements of the midgut prevented any further observations of the migrating cells.

To trace cell migration with a lipophilic dye, guts were dissected in 3 ml L15 medium in an incubation chamber with a glass cover slip as bottom. Then, 20 µl of DiO (1 µg 3,3´-dioctadecycloxacarbocyanine perchlorate, Molecular Probes, dissolved in 0,5 ml 100% Ethanol) were applied to the incubation chamber, so as to form a thin DiO layer on the surface of the L15 medium. The anterior parts of the guts were carefully manipulated several times to the level of this layer, which resulted in intense staining of the MG neurons and parts of the midgut cells. The DiO layer on the surface was removed with filter paper and the guts were washed several times in L15 medium.

Each preparation was placed at a temperature of 33°C in a PTC-10npi warming heater on the stage of a Zeiss Axiovert 35 fluorescence microscope equipped with FITC filters. A 10% neutral density filter was used to attenuate the excitation light emitted by the Xenon lamp. Images were captured at 20 minute intervals for a minimum of 2 hours with a Hamamatsu 2400 SIT camera linked to Simple PCI computer software (Hamamatsu, Hamamatsu City, Japan) which also controlled the opening of the shutter in the light path of the fluorescence excitation.

3. RESULTS

3.1. cGC and NOS activity in the developing enteric midgut plexus

During the development of the enteric nervous system of *Manduca*, NO-induced cGMP-IR has been reported in migrating nerve cells (Wright et al., 1998). To determine whether NO sensitive sGC activity is also present in MG neurons of the grasshopper embryo, I treated embryonic stages with the NO donor SNP to stimulate sGC activity and then immunostained the preparations with an antiserum that specifically recognizes cGMP (De Vente et al., 1987; Tanaka et al., 1997). These results revealed that sGC activity in MG neurons is indeed developmentally regulated, coincident with their migration on the midgut surface (Fig. 2, 3, 4). Prior to the onset of migration (up to 60% of embryonic development) no cGMP-IR was detectable in MG neurons. At 62% of embryonic development (% E), the MG neurons accumulate near the foregut-midgut boundary and start to migrate posteriorly (Fig. 2, 4A,B). At this stage, first cGMP-IR became visible and persisted during the following stages. The onset of detectable levels of cGMP-IR within the MG neurons occurred relatively rapidly. Between 60% and 65% E all migrating MG neurons acquired substantial levels of immunoreactivity (Fig. 2, 4F). The MG neurons exhibited cGMP-IR throughout the phase of migration (Fig. 2, 3, 4G) and continued to show high levels of anti-cGMP staining in the phase of lateral axon branching and the formation of terminal processes on the midgut (Fig. 2, Fig. 4H). The immunoreactivity was visible in the cell bodies and processes of the neurons (Fig. 4C, D). Between 90% and 95% E the midgut plexus acquires a mature branching pattern, accompanied by a rapid decrease in the percentage of cGMP expressing neurons (Fig. 2, 3).

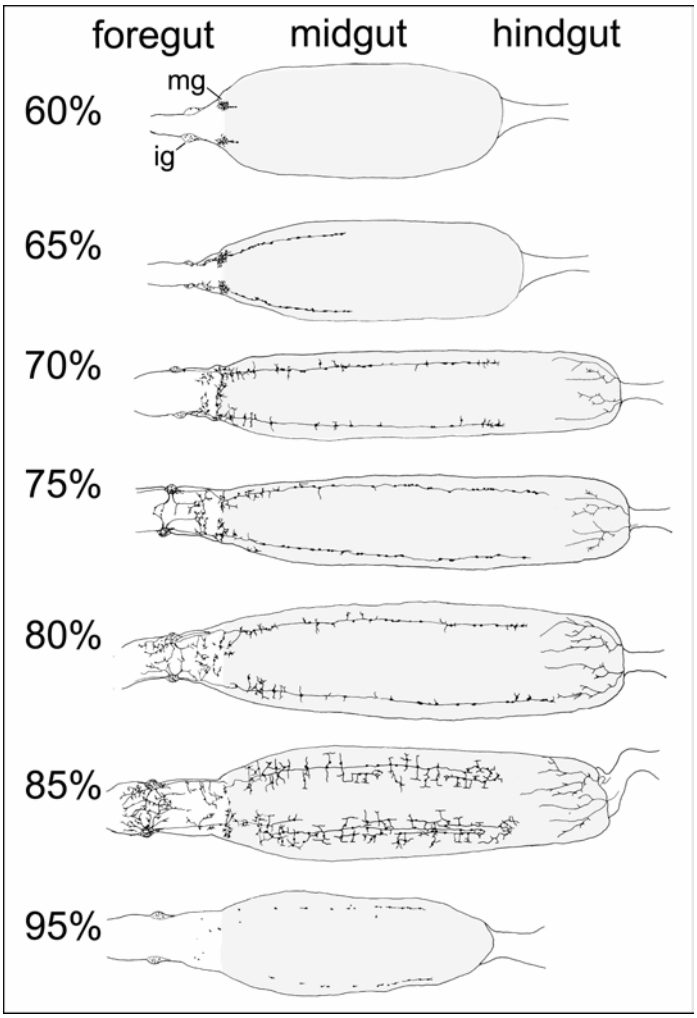

Fig. 2 NO induced cGMP-Immunoreactivity of MG neurons during midgut plexus development

Fig. 2: To show the distribution of NO-sensitive sGC activity in the developing ENS at specific stages of embryogenesis, guts were incubated with SNP and then immunostained with an anti-cGMP antiserum. Images were drawn from individual preparations. Each panel shows a dorsal view of the embryonic gut; ingluvial ganglion (ig), the midgut is marked in gray. For the sake of clarity, the caeca were not shown. At 55% E, almost no cGMP-IR was found in the premigratory population of MG neurons. When migration starts at about 60% E, all migrating MG neurons showed strong levels of anti-cGMP staining. The MG neurons exhibited cGMP-IR throughout the phase of migration (60% - 70% E) and continue to show high levels of anti- cGMP staining in the phase of lateral axon branching and the formation of terminal processes on the midgut musculature (70% - 85% E). When the midgut plexus acquired a mature innervation pattern (90% and 95% E), there was a rapid decrease in the amount of cGMP-positive MG neurons. (Scale bar: 200 µm).

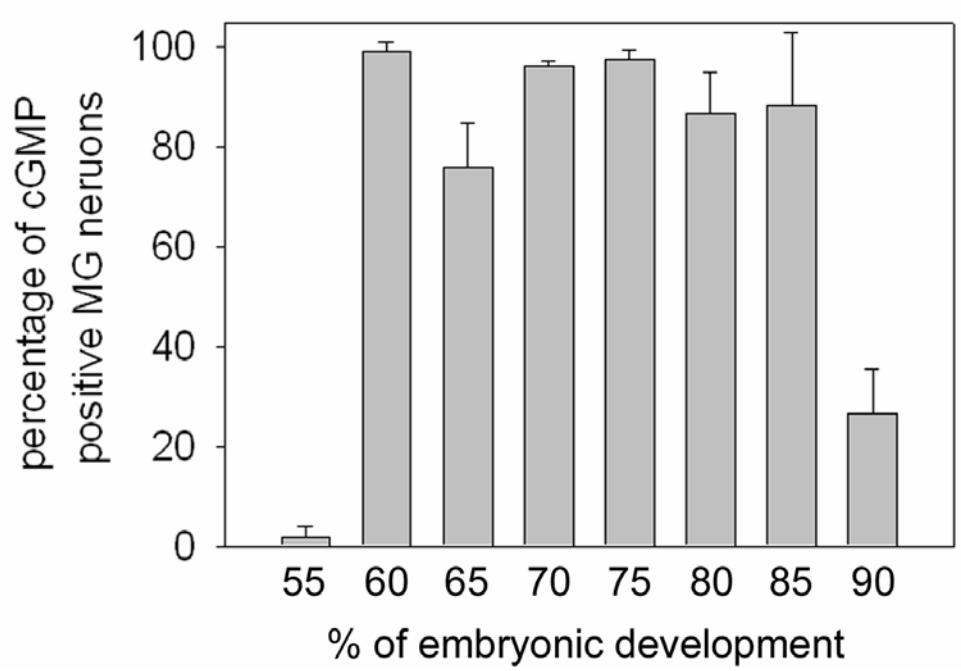

Fig. 3 Appearance of NO-induced cGMP-IR in MG neurons
The total number of MG neurons was calculated from anti-acetylated α-Tubulin staining which labels all MG neurons. The histogram shows the percentage of cGMP-positive MG neurons during different embryonic stages. The mean values and s.e.m. were calculated for each developmental stage (N = 5).

Cells originating within the terminal ganglion of the CNS innervate the posterior gut through the hindgut nerves. NO-sensitive sGC activity was also present in the branches of the hindgut nerves (Fig. 2). The leading processes of these abdominal neurons exhibited inducible cGMP-IR at the time of their initial growth onto the hindgut and remained immunopositive throughout their subsequent elaboration of processes on the hindgut and posterior midgut musculature.

The presence of NO-inducible sGC activity in the migrating MG neurons suggested that NOS expression might also be associated with the developing midgut plexus. I identified potential sources of NO near the MG neurons, using NADPH-diaphorase staining of formalin-fixed embryonic guts as a histochemical marker for NOS. The blue precipitate of the diaphorase staining is confined to a subset of the midgut cells (Fig. 4E).

Remarkably, the NADPHd staining was also developmentally regulated. Diaphorase staining of the midgut cells was visible during a developmental period ranging from about 60% to 95% of development. Between 60% to 65% E, diaphorase activity was restricted to an intense band of staining on the midgut surface, adjacent to the foregut-midgut boundary, which corresponded to the position of the migrating MG neurons. In later stages, discrete NADPH-diaphorase positive cells were found evenly distributed over all areas of the midgut epithelium.

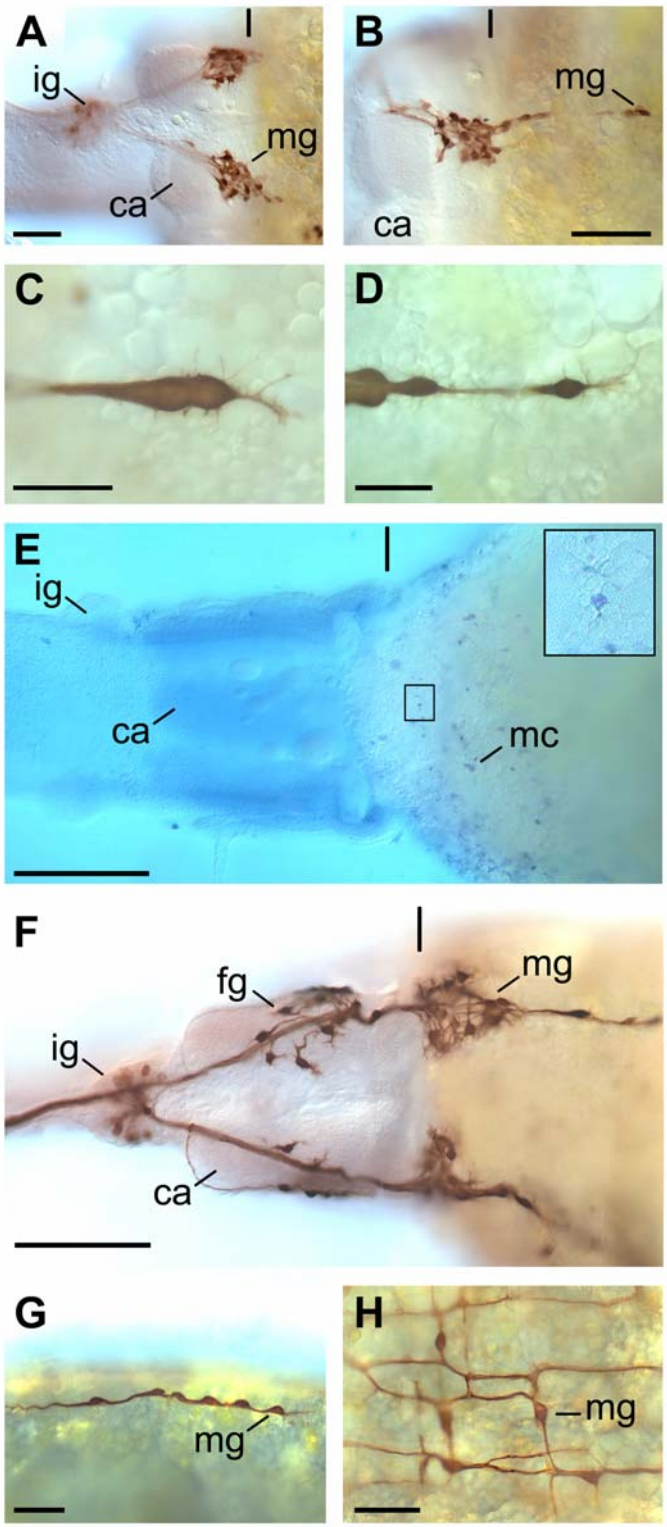

Fig. 4 **Developmental expression of NO induced anti-cGMP expression in MG neurons and NADPH diaphorase staining of the midgut epithelium**

Figure 4: (A) At about 60% of embryonic development (% E) the MG neurons (mg) formed a cellular packet at the foregut-midgut boundary (vertical line indicates boundary). Caeca (ca) were not stained. At this stage, all MG neurons began to show strong anti-cGMP staining. In the ingluvial ganglion (ig), some neurons expressed sGC activity (lateral view). (B) Between 60% – 65% E the first MG neurons started to migrate posteriorly on the midgut surface. All MG neurons showed high levels of anti-cGMP staining. (C and D) Between 60 – 65% E, NO-sensitive sGC activity was expressed in the cell body and the advancing processes of the leading MG neurons. (E) At 60% E first NADPH-diaphorase staining was present in distinct cells located in all areas of the midgut. The insert of the designated area shows an example of NADPH diaphorase positive cellular staining. The first appearance of the diaphorase staining was coincident with the onset of MG neuron migration (compare to A and B). (F) Anti-cGMP-IR at 65% E; lateral view. At this stage, anti-cGMP IR was present in cells of the ingluvial ganglion, the enteric nerves and the foregut neurons (fg). Some of the midgut neurons migrated laterally to form a nerve ring near the foregut-midgut boundary. (G) At 70% E, the MG neurons were still migrating posteriorly. The leading as well as the following neurons of one migratory pathway showed strong cGMP-IR. (H) During the phase of lateral neurite branching and the formation of terminal processes on the midgut musculature, the MG neurons continued to exhibit strong cGMP-IR. These photomontages were compiled from several focal planes. (Scale bars: A + B, 50 µm; C + D, 20 µm; E + F, 200 µm; G + H, 25 µm).

3.2. Nitric oxide/cGMP/PKG signalling is essential for MG neuron migration

The appearance of inducible cGMP-IR within the migrating MG neurons, and the presence of NADPH-diaphorase staining in a subpopulation of midgut cells, suggested that the transcellular activation of NO-sensitive sGC might be involved in MG neuron migration. To examine a potential role of the NO/cGMP pathway during the development of the midgut plexus, I cultured whole grasshopper embryos for 24 hours in neurochemicals known to affect this signalling pathway (Fig. 1).

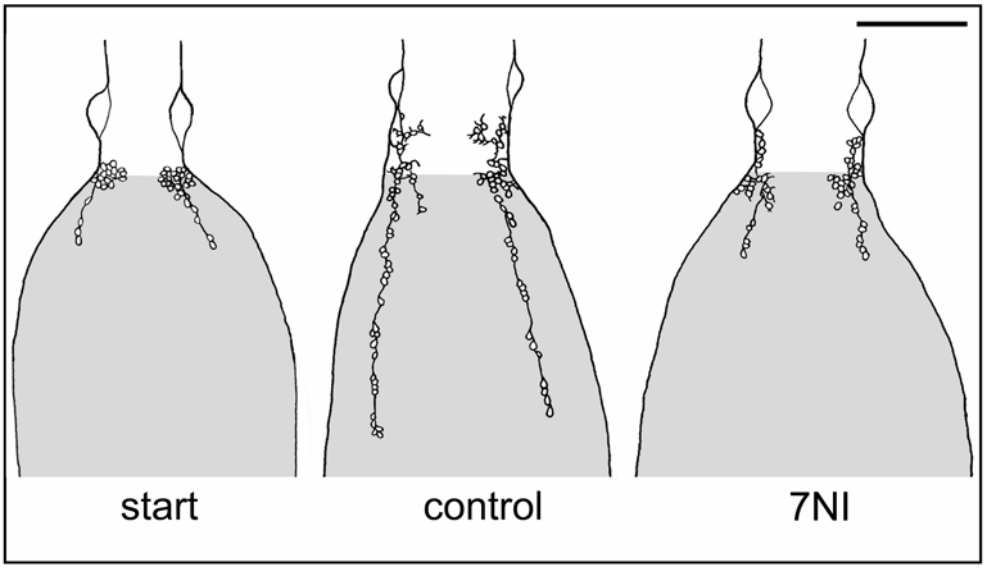

FIg. 5: NOS inhibitor 7NI blocks MG neuron migration
Images were drawn from individual guts that were stained with anti-α-tubulin antiserum. At the beginning of the experiment, MG neurons migrated not more than 40 µm on the midgut surface (start). Normal MG neuron migration occurred under culture conditions in which the culture medium contained the vehicle DMSO (control). MG neuron migration was inhibited in an embryo that was exposed to 500 µM 7NI. (Scale bar: 200 µm).

Cultured embryos were exposed to neurochemicals that elevated cGMP levels. I used protoporphyrin IX free acid which stimulates sGC independently of NO (Wollin et al., 1982). The membrane-permeable 8Br-cGMP was used to raise cGMP levels directly. Alternatively, embryos were treated with compounds that inhibit the enzyme activity of NOS, sGC or PKG.

When embryos at 62% of development, a stage at which the MG neurons initiate their migration, were allowed to develop in culture for 24 hours, I observed that MG neuron migration proceeded normally. In contrast, migration was significantly reduced in embryos that were exposed to the NOS inhibitor 7NI (500 µM) (Fig. 5, 6).

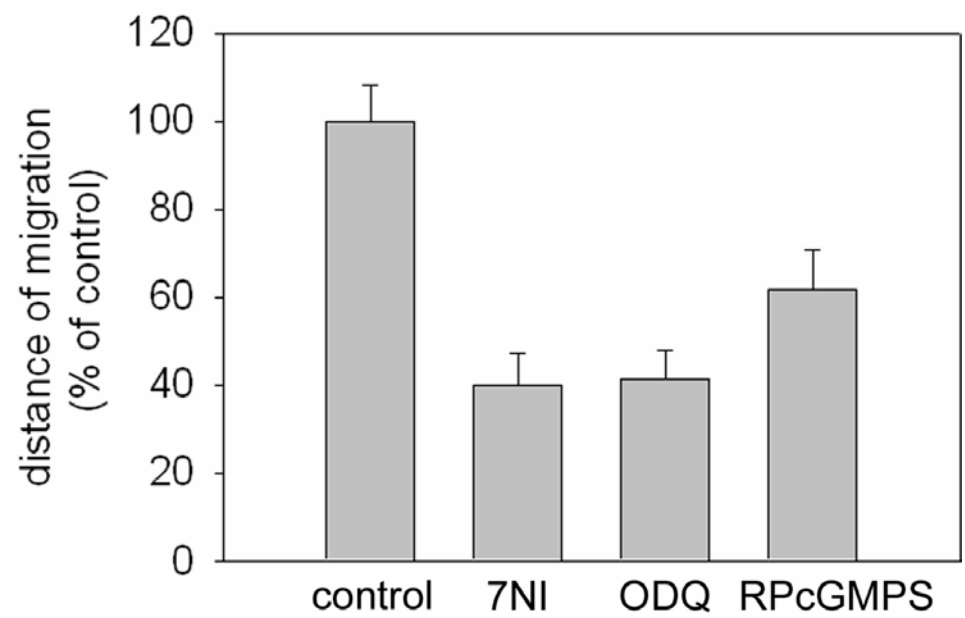

Fig. 6 Blocking of MG neuron migration by enzyme inhibitors of the NO/cGMP/PKG pathway
Histograms show the distance migrated by the leading MG neuron on the midgut. (A) After 24 hours, normal migration of MG neurons was found in cultured control embryos (N = 21). MG neuron migration was significantly reduced in the presence of 500 µM 7NI (N = 21), 200 µM ODQ (N = 20), and 50 µM RPcGMPS (N = 21). ** $P < 0.005$; *** $P < 0.001$.

Selective inhibition of NOS was provided by the exposure to 7NI (Doyle et al., 1996), blocking of sGC activity was achieved by using ODQ (Garthwaite et al., 1995), and for inhibition of the PKG I used RPcGMPS (Layland et al., 2002) (Fig. 1).Neither SNP (500 µM), protoporphyrin IX free acid (500 µM) nor 8Br-cGMP (500 µM) had any effect on the normal migration of the MG neurons (data not shown). In contrast, neurochemicals that inhibited the NO/cGMP pathway significantly affected the migration of MG neurons. To test for an involvement of the target enzyme sGC in MG neuron migration, embryos were cultured with the specific inhibitor ODQ. This treatment reduced MG neuron migration in a concentration dependent manner (Fig. 7).

Embryo culture in the presence of 200 µM ODQ caused a significant reduction in MG neuron migration similar as the 7NI treatment (Fig. 6, 7). A potential downstream effector proteins for cGMP signalling are cGMP-dependent protein kinases (Wang and Robinson, 1997). In embryos treated with the specific inhibitor RPcGMPS, which irreversibly binds to PKG, MG neuron migration was significantly reduced compared to control-treated animals (Fig. 6).

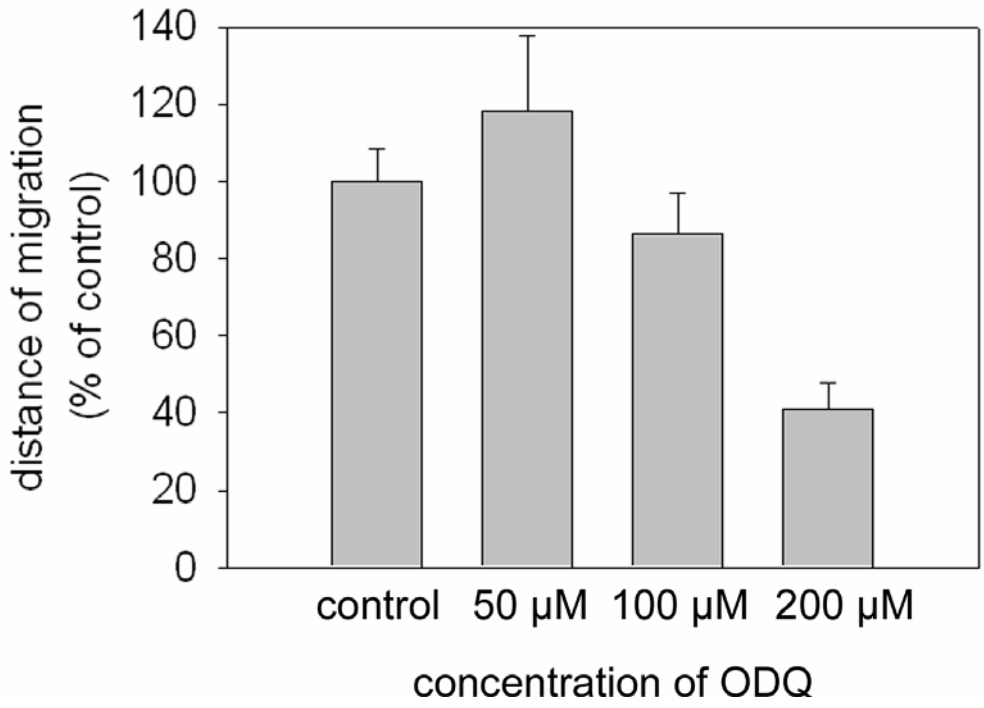

Fig. 7 Quantification of sGC inhibition by ODQ. ODQ reduced MG neuron migration in a concentration-dependent manner
Fig. 7: Histograms show the distance migrated by the leading MG neuron on the midgut. Normal migration of MG neurons was highly significantly inhibited in embryos that were cultured in the presence of 200 µM ODQ (control, N = 21; 200 µM, N = 20; 100 µM, N = 20; 50 µM, N = 24). *** $P < 0.001$.

3.3. NO- and cGMP-analogs rescue disruptive effects of NOS and sGC inhibitors

Both ODQ and 7NI produce similar effects in disrupting MG neuron migration. To test if the disruptive effects of both enzyme inhibitors can be reversed by adding activators of NO/cGMP signalling, I treated embryos in culture with ODQ plus 8Br-cGMP (Fig. 8, 9A) as well as with 7NI plus protoporphyrin IX free acid (Fig. 9B). Embryos cultured in the presence of 200 µM ODQ showed a significant reduction of migration. However, adding 500 µM of the membrane permeable 8Br-cGMP led to a complete recovery of the normal MG neuron migration (Fig. 8, 9A).

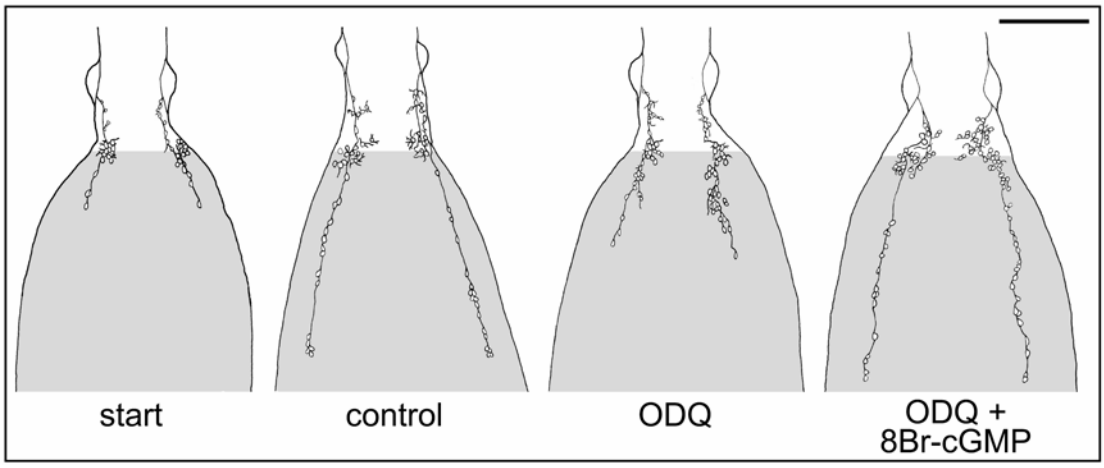

Fig. 8 The blocking effects of the sGC inhibitor ODQ on MG neuron migration was rescued by the addition of 8Br-cGMP

Images were drawn from individual guts that were stained with anti-α-tubulin antiserum. At the beginning of the experiment, MG neurons had not migrated more than 40 µm on the midgut surface (start). The next drawing shows normal MG neuron migration after 24 hours incubation under control culture conditions (control). MG neuron migration was reduced in an embryo that was exposed to 200 µM ODQ (ODQ). The inhibitory effect of ODQ was rescued by the addition of membrane permeable 8Br-cGMP (ODQ 200 µM + 8Br-cGMP). (Scale bar: 200 µm).

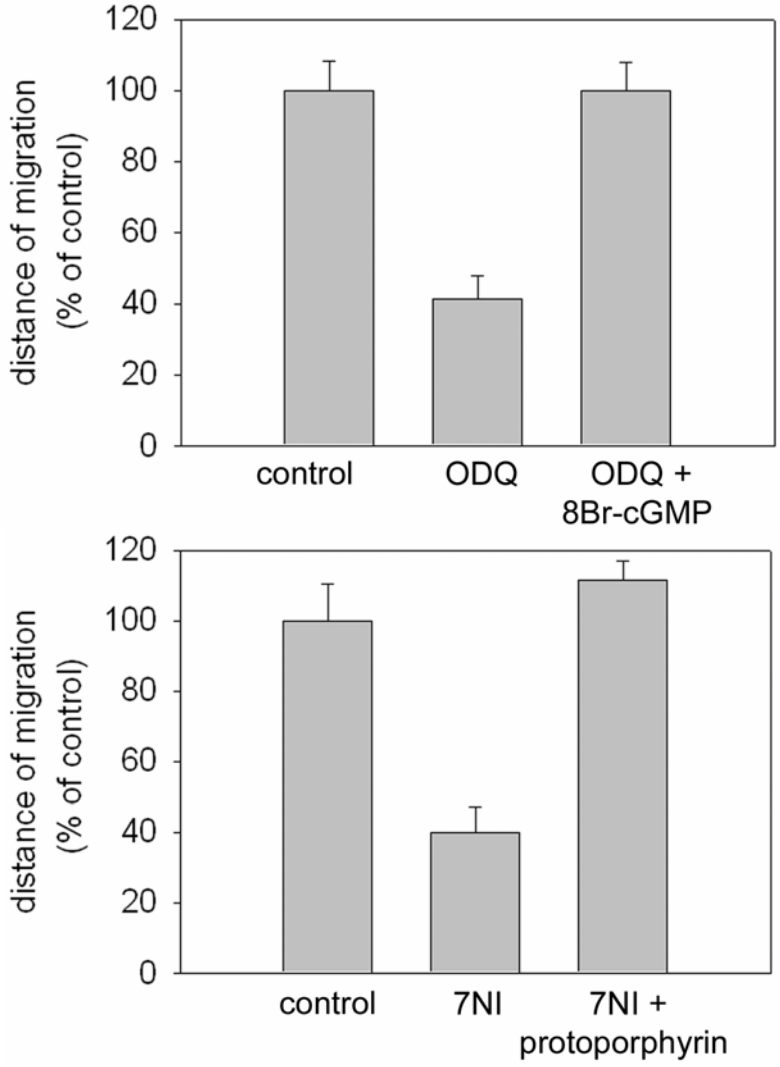

Fig. 9 Quantification of the blocking of MG neuron migration by the sGC inhibitor ODQ and the NOS inhibitor 7NI

Figure 9: Histograms show the distance migrated by the leading MG neuron on the midgut.

(A) Incubation in 200 µM ODQ resulted in a significant reduction of the migratory distance (ODQ; N = 20) as compared to cultured control embryos (control; N = 21). This disruptive effect of ODQ could be rescued by the addition of 8Br-cGMP such that the MG neurons again covered the normal distance (ODQ + 8Br-cGMP; N = 20).

(B) Histogram shows that incubation in 500 µM 7NI resulted in a significant reduction of the migratory distance (7NI; N = 21) as compared to cultured control embryos (control). This disruptive effect of 7NI could be rescued by the addition of 1 mM protoporphyrin IX free acid so that the MG neurons again covered the normal distance (7NI + protoporphyrin; N = 20). *** $P < 0.001$.

As already described, embryos cultured in the presence of 500 µM 7NI showed a significant inhibition of normal MG neuron migration. Again, addition of the sGC-activating protoporphyrin IX free acid (1mM) reversed the inhibitory effect of 7NI on cell migration and resulted in a normal migration pattern (Fig. 9B). These results show that application of membrane-permeant cGMP and an NO-analogue rescue the defects caused by inhibiting endogenous NO/cGMP signalling.

3.4. cAMP/PKA signalling inhibits MG neuron migration

The appearance of inducible cGMP-IR within the migrating MG neurons suggested that cyclic nucleotides might play a role in MG neuron migration. To examine a potential role of the cAMP/PKA pathway during the development of the midgut plexus, I cultured whole grasshopper embryos for 24 hours in neurochemicals known to affect this signalling pathway (Fig. 1).

Cultured embryos were exposed to neurochemicals that elevated cAMP levels. I used Forskolin (100 µmol), which stimulates the adenylat cyclase (AC) to produce cAMP (Sun et al., 2002). The membrane permeable SPcAMPS (50 µmol) was used to activate PKA directly. Alternatively, embryos were treated with RPcAMPS (25 µmol) which selectively inhibits PKA activity. When embryos at 62% of development, a stage at which the MG neurons initiate their migration, were allowed to develop in culture for 24 hours, I observed that MG neuron migration proceeded normally (Fig. 10).

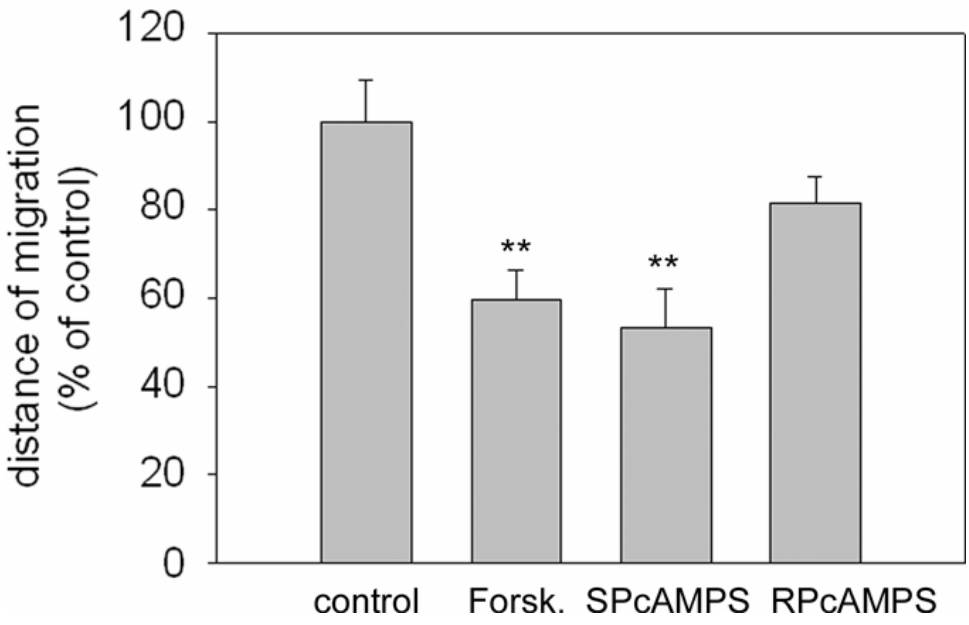

Fig. 10 Blocking of MG neuron migration by enzyme activators and inhibitors of the cAMP/PKA pathway

Histograms show the distance migrated by the leading MG neuron on the midgut. (A) After 24 hours, normal migration of MG neurons was found in cultured control embryos (N = 21). MG neuron migration was significantly reduced in the presence of 100 µM Forskolin (N = 20), 50 µM SPcAMPS (N = 21). In contrast, 50 µM RPcAMPS had no significant effect on migration (N = 21). ** $P < 0.005$.

In contrast, neurochemicals that activated the cAMP/PKA pathway affected the migration of MG neurons. Migration was significantly reduced in embryos that were exposed to the AC activator Forskolin plus the phosphodiesterase inhibitor IBMX (Fig. 10). A possible target of cAMP is the receptor enzyme PKA. In embryos treated with the specific activator SPcAMPS, which irreversible binds to PKA, MG neuron migration was significantly reduced compared to control-treated animals (Fig. 10). Whereas, the PKA inhibitor RPcAMPS had no effect on the normal migration of the MG neurons compared to control-treated embryos (Fig. 10).

3.5. F-actin cytoskeleton in MG neuron cell culture

Cytoskeletal organization plays an important role in mediating cell migration (Hassid et al., 1999). Increased cell migration is associated with actin filament dissociation in vascular smooth muscle cells, endothelial cells, and fibroblasts (Ichijima et al., 1993; Richman and Regan, 1998; Brown et al., 1999; Amieux et al., 2002). To examine the dynamic regulation of the F-actin cytoskeleton in MG neurons under the influence of different neurological pharmaca known to affect the cGMP/PKG or the cAMP/PKA signalling pathway, I used a combined embryo culture/cell culture system. Embryos of a stage where MG neurons initiate migration were incubated in different pharmacological substances for 24 hours (Fig. 1). Subsequently, the guts were dissected out, the MG neurons were blotted on a coated glass coverslip and immediately fixed. This method was supposed to demonstrate the cells in an almost *in vivo* state.

To visualize the actin cytoskeleton in the MG neurons, F-actin was labeled with phalloidin. To verify the neuronal identity of the blotted cells I used an antiserum against horseradish peroxidase which is known to stain a carbohydrate epitope, selectively expressed by membrane proteins on the surface of insect neurons (Jan and Jan, 1984).

I found a significant difference between embryos treated with pharmacological reagents and control cultured embryos with respect to the F-actin distribution in MG neurons. In control experiments, MG neuron migration proceeded normally (see above). Under these physiological conditions, I observed an actin filament disassembly in most of the neurons. In these cells, F-actin staining was exclusively found in the neurites and the cell cortex, whereas, in the cell body, F-actin staining was almost absent (migratory phenotype). In these control experiments, 70% of the MG neurons showed the migratory phenotype. Whereas, in 30% of the neurons, F-actin fibres were present in the somata (Fig. 11, 13).

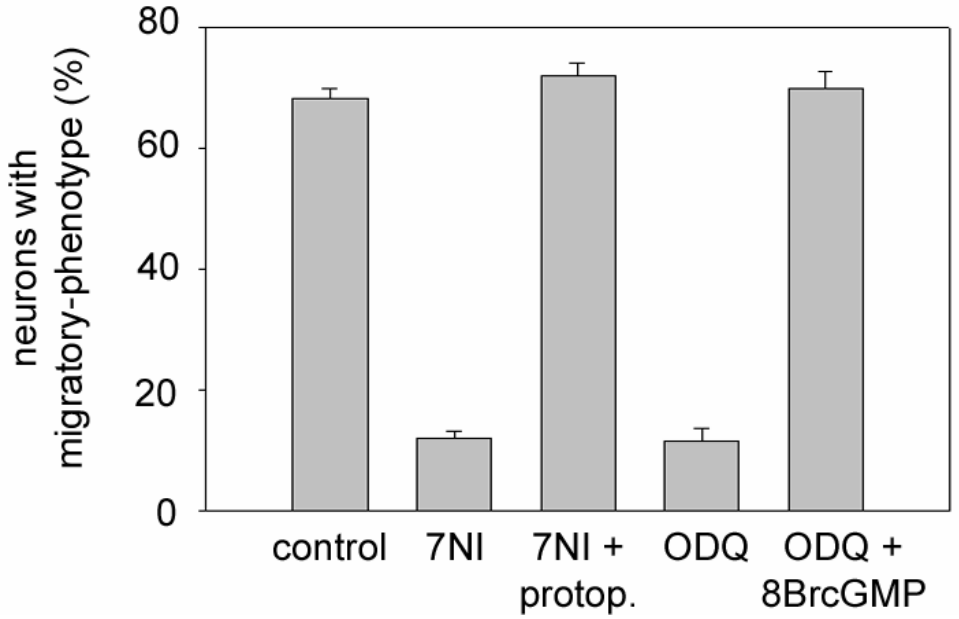

Fig. 11 Quantification of the actin cytoskeleton reorganization caused by enzyme inhibitors that could be reversed by the addition of enzyme activators of the NO/cGMP pathway

Fig. 11: Histograms show the percentage of MG neurons showing a migratory phenotype after incubation in enzyme activators or inhibitors of the NO/cGMP pathway. For this experiment, a combined embryo culture/cell culture system approach was used. The actin cytoskeleton was visualized with Phalloidin staining. After 24 hours incubation, a high percentage of MG neurons show the normal migratory phenotype in cultured control embryos (control). In contrast, a significantly smaller number of neurons from embryos cultured in the NOS inhibitor 7NI (500 µM). The disruptive effect of 7NI on the actin cytoskeleton could be rescued by the addition of 1 mM protoporphyrin IX free acid (7NI + protoporphyrin; N = 20). A significantly smaller number of neurons from embryos cultured in the sGC inhibitor ODQ (200 µM) showed the migratory phenotype. This disruptive effect of ODQ on the actin cytoskeleton organization could be rescued by the addition of 500 µM 8Br-cGMP (ODQ + 8Br-cGMP). ** $P < 0.005$; *** $P < 0.001$.

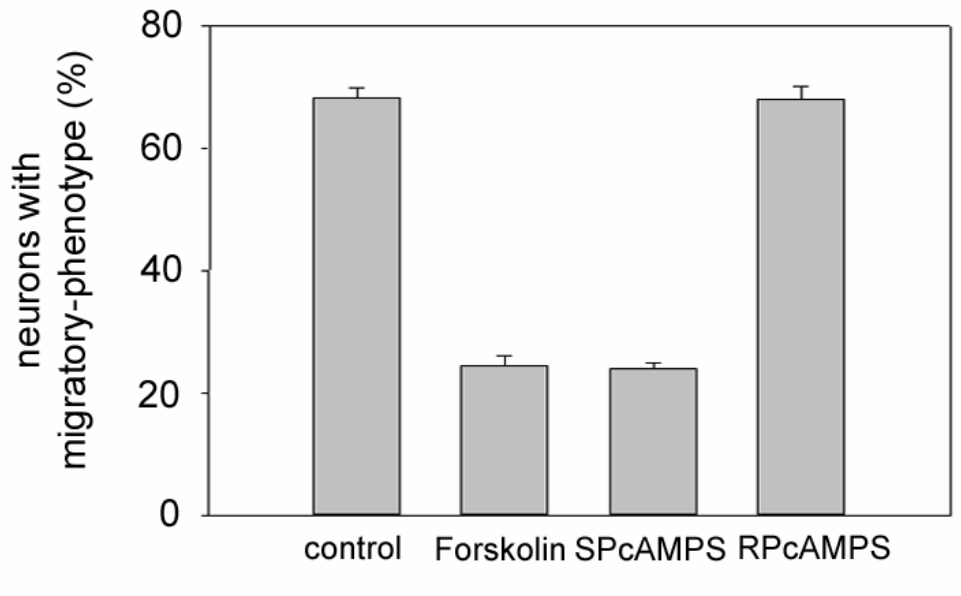

Fig. 12 Quantification of the actin cytoskeleton reorganization caused by enzyme activators and inhibitors of the cAMP/PKA pathway

Fig. 12: Histograms show the percentage of MG neurons showing a migratory phenotype after incubation in enzyme activators or inhibitors of the cAMP/PKA pathway. For this experiment, a combined embryo culture/cell culture system approach was used. The actin cytoskeleton was visualized with Phalloidin staining. After 24 hours incubation, a high percentage of MG neurons show the normal migratory phenotype in cultured control embryos (control). In contrast, a significantly smaller number of neurons from embryos cultured in the AC activator Forskolin (X µM,) or the PKA activator SPcAMPS (50 µM) showed the migratory phenotype. The PKA inhibitor RPcAMPS (50 µM) had no significant effect on the actin cytoskeleton organization. ** $P < 0.005$.

In contrast, in MG neurons treated with the NOS inhibitor 7NI or the sGC inhibitor ODQ, prominent F-actin staining was present in the somata (non-migratory phenotype). The F-actin fibers formed a dense net of actin-bundles in all parts of the soma. This phenotype was shown by 88% (7Ni) or 90% (ODQ) of the neurons. In only 12% 10% of the cells, actin-filaments were absent from the somata. In MG neurons treated with the sGC inhibitor ODQ, 90% of the neurons showed the non-migratory phenotype with a dense net of F-actin fibers in all parts of the soma. In only 10% of the cells, actin filaments were absent from the somata (Fig. 11).

In order to test if the effects of 7NI can be reversed by adding the NOS activator protoporphyrin IX free acid, the embryos were incubated in 7NI plus protoporphyrin IX free acid which reversed the effect of 7NI (Fig. 11). In this rescue experiments, 70% of the MG neurons showed the migratory phenotype. Whereas, in only 30% of the neurons, F-actin fibres were found in the somata. Similarly, the effect of ODQ on the actin cytoskeleton could be rescued by the addition of membrane-permeant 8Br-cGMP. In this rescue experiments, 94% of the MG neurons showed the migratory phenotype. Whereas, in only 6% of the neurons, F-actin fibres were found in the somata (Fig 11).

Similarly, neurochemicals that activated the cAMP/PKA pathway affected significantly the F-actin distribution in MG neurons. MG neurons treated with the AC activator Forskolin in the presence of the unspecific phosphodiesterase inhibitor IBMX (which prevents cAMP degradation) showed dense F-actin staining in the somata (75% of the neurons). Whereas, Forskolin alone had no effect on normal migration of the MG neurons (data not shown). Furthermore, in MG neurons that where incubated in the PKA activator SPcAMPS, the same prominent F-actin staining of the somata was present in 60% of the neurons (Fig. 11).

Neurons incubated in the PKA inhibitor RPcAMPS showed no differences in the F-actin distribution, compared with control treated neurons (Fig. 11).

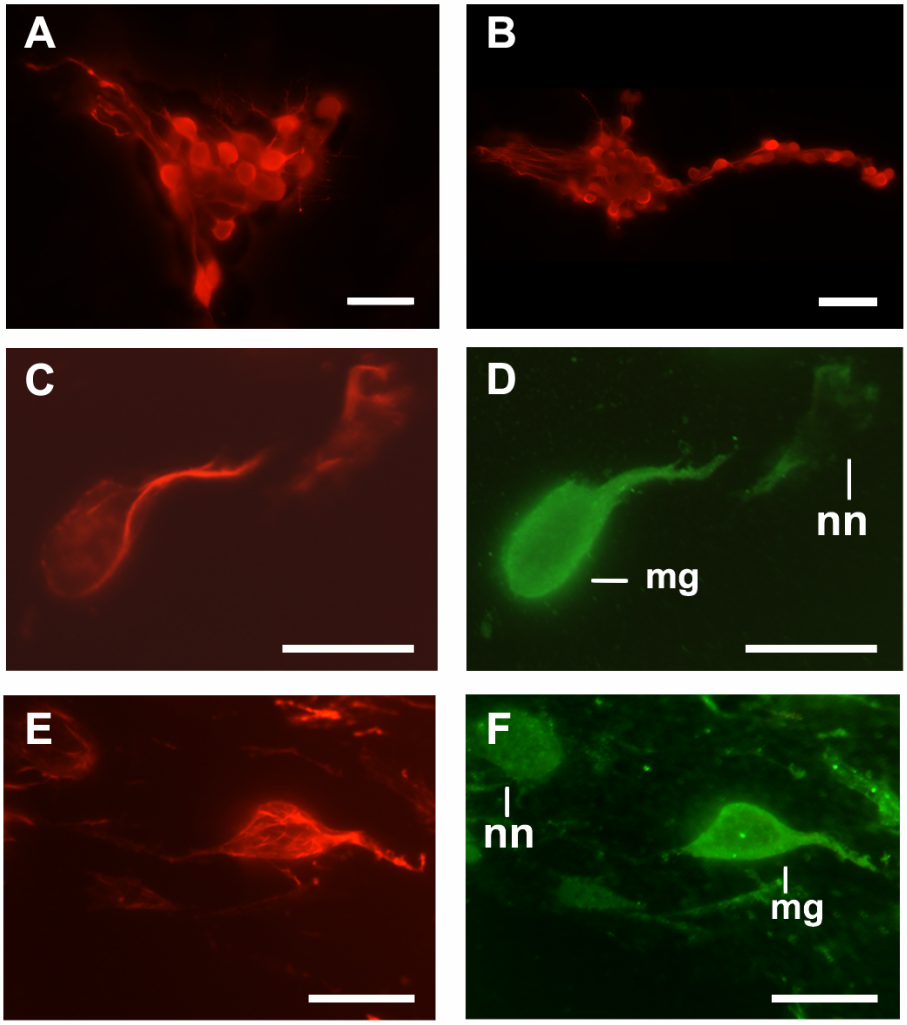

Fig. 13 NO induced anti-cGMP expression in cultured MG neurons and F-actin staining in combined embryo culture/cell culture experiments.
(A + B) After 24 hours in cell culture the MG neurons show prominent NO-induced cGMP-IR. (C) In contol experiments, the MG neurons show the normal migratory phenotype with F-actin staining in the neurite and without actin fibres in the soma (migratory-phenotype). (D) The same neuron as in (C): Co-staining with anti-HRP antiserum reveals the neuronal identity of the cell. (G) When the embryos were cultured in ODQ, a neruochemical that inhibits migration, the MG neruons showed a non-migratory phenotype wit prominent Phalloidin-staining in the somata. (H) Anti-HRP staining reveals the neuronal identity of the cells in (G + I). (Scale bars: A 20 µm; B, 30 µm; C - F, 10 µm).

NO-induced cGMP-immunoreactivity in cultured MG neurons

Treatment with NO donors induced cGMP-IR in the MG neurons *in situ* (see above). To determine whether NO sensitive sGC activity is present in cultured MG neurons, I treated MG neurons cultured for 24 – 48 hours with the NO donor SNP to stimulate sGC activity and then immunostained the cells with an antiserum that specifically recognizes cGMP (De Vente et al., 1987). This experiment revealed that sGC activity is present in MG neurons under cell culture conditions. In cell culture, cGMP immunoreaktivity is found in all MG neurons, similar to embryo culture experiments (Fig. 13). Inducible cGMP-IR could be seen throughout the somata and neurites of the MG neurons. When sGC activity was blocked with the specific inhibitor ODQ (200 µmol) anti-cGMP staining was completely abolished (not shown).

3.6. Time-lapse video microscopy of living MG neurons

To investigate the normal migratory behavior of individual cells *in situ*, the motility of fluorescence-labelled MG neurons was analyzed under time-lapse video microscopy (Fig. 10). The movements of DiO-stained MG neurons were followed under low-light level illumination on intact midguts for 2 hours showing that the average velocity migrated by each cell (N = 5) was approximately 12 µm/hour (Fig. 10).

There are several possibilities how disruption of cGMP signalling might affect directed cell migration such as simply stopping, disorientation of the migratory pathway, or in the extreme, reversal of normal direction of movement. To test these possibilities, the migrating neurons were exposed to 200 µM ODQ and tracked for two hours (Fig.14). All neurons immediately stopped migration under the influence of ODQ. No misrouting or reversion of the movement direction was observed.

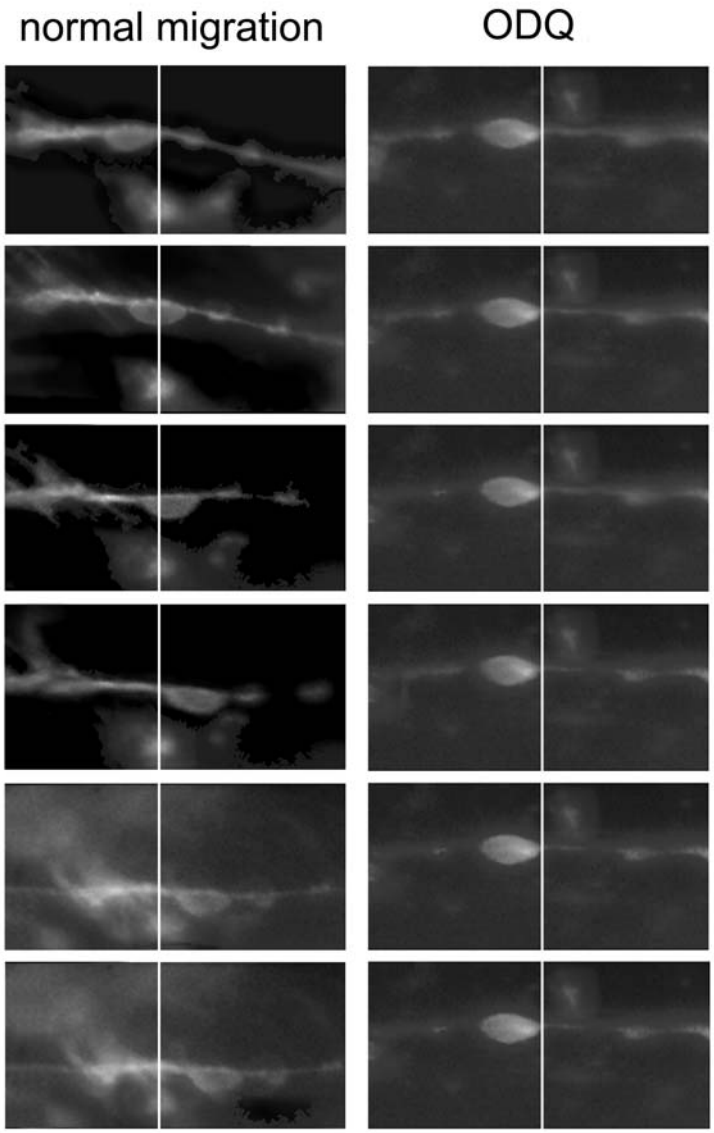

Fig. 14 Time-lapse video microscopy of living MG neurons migrating on the midgut

Examples show DiO-labelled MG neurons from embryos staged between 64% and 68% E. Time intervals between the images are 20 minutes. On average, MG neurons migrated 12 µm/hour under normal conditions (N = 5). To examine the effects of inhibiting sGC on individual neurons, the preparation was incubated in culture medium containing 200 µM ODQ (N = 5). Under these conditions, there was no misrouting of the neurons and they completely stopped migrating. (Scale bar: 10 µm).

4. DISCUSSION

In this paper, I have provided evidence that NO/cGMP signalling is essential for the regulation of neuronal migration in the developing ENS of the grasshopper. I found that blocking of endogenous NO synthesis by the NOS inhibitor 7NI disrupts migration of the MG neurons (Fig. 5, 6). Treatment with ODQ, a specific inhibitor of sGC (Boulton et al., 1995) also prevented the MG neuron migration in a dose-dependent manner (Fig. 6, 7). In embryos treated with the specific PKG inhibitor RPcGMPS (Layland et al., 2002), MG neuron migration was significantly reduced (Fig 6). This effect suggests that cGMP might influence migration via activating PKG.

It is rather unlikely that the pharmacological effects on MG neuron migration are an artefact of the embryo culture, since in control cultured embryos, migration proceeded normally. Intriguingly, the disruption of MG neuron migration caused by inhibiting NO production or cGMP synthesis could be rescued by exogenous application of membrane-permeant cGMP (Fig. 8, 9A) and pharmacological stimulation of sGC (Fig. 9B), suggesting that *in vivo* a certain level of cGMP is necessary for MG neuron migration. In a similar pharmacological approach, NO and cGMP have been shown as positive regulators of axonal elongation of pioneer neurons in grasshopper embryos (Seidel and Bicker, 2000).

4.1. Immuno- and histochemical detection of sGC and NOS

The hypothesis that NO/cGMP signalling is necessary for MG neuron migration receives additional support from cytochemical stainings. Treatment with a NO donor induced cGMP-IR in the MG neurons in the developing ENS of *Locusta migratoria*. Moreover, the timing of sGC activity coincides exactly with periods of neuronal motility as well as axonal outgrowth. At the onset of their migration on the midgut surface, first detectable levels of cGMP-IR within the MG neurons became apparent. The MG neurons exhibited cGMP-IR throughout the phase of migration and continued to show high levels of anti-cGMP staining in the phase of lateral axon branching and the formation

of terminal processes on the midgut musculature (Fig. 2, 3, 4). When the midgut plexus acquired a mature morphology, a rapid decrease in the amount of cGMP-positive MG neurons occurred (Fig. 2, 3). Thus, NO-induced sGC activity in MG neurons is developmentally regulated and correlates with their migratory phase.

Moreover, I identified a potential source of endogenous NO in immediate vicinity to the migrating MG neurons. A subpopulation of the midgut cells stain for NADPH-diaphorase, a histochemical marker for NOS (Dawson et al., 1991; Vincent and Kimura, 1992) (Fig. 4E). Again, the first appearance of NADPH-diaphorase activity in the midgut epithelium exactly coincides with the onset of MG neuron migration. The diaphorase staining persisted throughout the phase of MG neuron migration and the phase of terminal branch formation.

NADPHd histochemistry following formaldehyde fixation is considered to be diagnostic for the presence of NOS containing cells (Dawson et al., 1991; Vincent and Kimura, 1992). In the nervous system of adult grasshoppers, measurements of NOS activity in cell homogenates of various regions do indeed correlate well with the biochemical determination of NADPHd activity and the histochemical staining pattern of NADPHd positive cells (reviewed in Bicker, 1998). Nevertheless, the results of the diaphorase method are subject to variations due to the fixation protocols and may even lead to false positive results (Ott and Burrows, 1999). However, using an antiserum that recognizes a highly conserved sequence of the different mammalian NOS isoforms, it has been shown that NOS-IR in somata does indeed co-localize with a NADPHd positive cell body cluster on double-stained cryosections of the antennal lobe (Bicker, 2001b). This finding supports the molecular identity of NADPH diaphorase and NOS enzymes in grasshopper tissue fixed according to the histological protocol of this paper. In the intact embryo there may be additional messenger molecules apart from NO that could activate sGC. For example the MG neurons may receive a carbon monoxide signal (Baranano and Snyder, 2001) from yet unidentified tissue sources.

In *Manduca*, Wright et al. (1998) found NO-sensitive sGC expression in a migratory subset of enteric neurons called EP cells which form the enteric midgut plexus similarly to the MG neurons. In contrast to the grasshopper, in *Manduca* there was no detectable NO source near the migrating EP cells and the EP cell migration on the midgut was not affected by pharmacological manipulation of NO/cGMP signalling. However, the inhibition of NOS and sGC results in a reduction of terminal synaptic branch formation of the enteric neurons (Wright et al., 1998). The different outcome of these experimental results might be due to species-specific differences in the development of the midgut plexus in the holometabolous *Manduca* and the hemimetabolous *Locusta*. Differences in the experimental procedures of animal culture, time of drug exposure, or concentration of the pharmacological agents may have also contributed to the different outcome of the cell migration experiments. For example, I found a strong inhibitory effect on cell migration of the sGC blocker ODQ at 200 µM (Fig. 6, 7), a concentration which has not been used in *Manduca*. Nonetheless, it should be stressed that in the grasshopper the ODQ effect could be fully rescued to normal migratory behavior by a cGMP analogue.

4.2. Cyclic nucleotide signalling influence migration

Moreover, I have provided evidence that the cyclic nucleotides cGMP and cAMP have antagonistic effects on the regulation of neuronal migration in the developing ENS. In addition to the finding that cGMP/PKG signalling is essential for normal MG neuron migration, I found that pharmacological elevation of cytosolic cAMP and direct stimulation of PKA results in an inhibition of normal migration (Fig. 10). In particular, using the embryo culture system, it could be shown that elevation of cytosolic cAMP levels by the AC activator Forskolin disrupts migration of the MG neurons. Treatment with SPcAMPS, a specific activator of PKA also inhibited normal MG neuron migration, indicating that cAMP influence migration via a PKA dependent mechanism, whereas, the specific inhibitor of PKA, RPcAMPS had no disruptive effect on MG neuron migration. It is unlikely that the pharmacological effects on MG neuron migration are an artefact of the embryo culture, since in control cultured embryos migration proceeded normally. Unfortunately, I could not localize the components of the cAMP/PKA signalling pathway in the migrating MG neurons. There is no suitable antiserum for the grasshopper nervous system to visualize the enzymes adenyly cyclase or PKA. For biochemical essay, that may

identify AC activity in the MG neurons or determine the phosphorylation status of PKA, the number of MG neurons available is far too small. In addition, the MG neurons are postmitotic cells and could not proliferate in cell culture. The grasshopper embryo is a convenient model system in which to study cell migration. However, there are several limitations, because the grasshopper is not accessible for genetic manipulations. Moreover, the majority of developmental studies in insects are performed in *Drosophila*. Thus, most of the commonly used antibodies are raised against *Drosophila* proteins and do not suit for the grasshopper embryo. Therefore, in this study, the function of cAMP/PKA signalling can only be deduced from the pharmacological data.

Effects of cGMP and cAMP on the actin cytoskeleton

Neuronal cell migration requires the dynamic regulation of the actin cytoskeleton which involves the polymerization and depolymerization, stabilization, and branching of actin filaments (Ballestrem et al., 1998). Thus, cell shape and cytoskeletal organization play important roles in mediating cell migration. I therefore examined the effects of cyclic nucleotides on cell morphology and actin filament organization. I examined the dynamic regulation of the F-actin cytoskeleton in MG neurons under the influence of different neurological pharmaca known to affect the cGMP/PKG or the cAMP/PKA signaling pathways. In the grasshopper, the visceral midgut cells are identified as a possible endogenous source of NO which stimulates the MG neurons to produce cGMP. In untreated control embryos, where migration proceeded normally, the migration-stimulatory effect of endogenous NO/cGMP signaling was associated with a rearrangement of the actin cytoskeleton of the MG neurons. Under these control conditions, a disassembly of actin fibres in the somata was observed. The F-actin staining was exclusively found in the neurites and the cell cortex, whereas in the cell body, F-actin staining was absent (called migratory phenotype). Thus, the migration-stimulatory effect of endogenous NO/cGMP signalling was associated with decreased levels of actin stress fibres in the somata of the neurons (Fig. 11, 13). In contrast, when the embryos were treated with the specific sGC inhibitor ODQ, I found a dense net of actin fibres in the somata in the MG neurons (called non-migratory phenotype) (Fig. 11, 13). The sGC inhibitor ODQ disrupts MG neuron migration in the embryo culture experiments. In conclusion, the inhibitory effect of ODQ on migration is related to a cytoskeletal rearrangement with dense actin fibres in the somata. Intriguingly, the effect of ODQ on the actin

cytoskeleton could also be reversed by the addition of membrane permeable cGMP resulting in a migratory phenotype, where actin fibres are absent from the somata, further supporting the involvement of cGMP as second messenger in this rearrangement (Fig. 11). Correspondingly, the inhibitory effects of cAMP/PKA signalling on MG neuron migration were also associated with actin cytoskeleton rearrangements (Fig. 12). MG neurons treated with the AC activator Forskolin and the PKA activator SPcAMPS exhibit prominent F-actin fibres in the somata. In conclusion, migrating MG neruons showed prominent F-actin fibres only in the leading edge of the cell and in the cell cortex, whereas stationary neruons had a dense net of actin fibres that spans the soma.

These results are in accordance with numerous studies, where a rearrangement of actin fibres was observed in the transition from a stationary to a motile state. Cell migration requires the dynamic regulation of the actin cytoskeleton (Ballestrem et al., 1998). The results of this thesis are consistent with the notion that actin fibres disassembly and migration may be causally related. For example, actin filament disassembly is associated with increased cell migration in both vascular smooth muscle cells and fibroblasts (Brown et al., 1999; Hassid et al., 1999). NO/cGMP signaling induces F-actin fibre dissociation in chondrocytes (Clancy et al., 1997). In postcapillary endothelial NO/cGMP signaling stimulates cell migration and the stimulatory effect of cGMP is associated with a disassembly of actin filaments (Ziche et al., 1997). Furthermore, in a mutant mouse model where basal activity of PKA is increased, fibroblast displayed an abnormal cytoskeleton with disorganized actin bundles within the cells in conjunction with a reduced ability of migration, whereas the wildtype cells only displayed sparse parallel actin fibres (Amieux et al., 2002).

Cyclic nucleotides influence neuronal navigation

A wealth of evidence from both invertebrates and vertebrates has shown that the growth cones integrate a multitude of extracellular guidance cues into attractive or repulsive steering decisions (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Song and Poo, 2001; Yu and Bargman, 2001; Dickson, 2002). However, the proper interpretation of these cues depends on the internal state of the growth cone. Several studies have demonstrated that attractive and repulsive guidance responses can be switched from one to the other by cyclic nucleotide

concentrations in the growth cone. Cell culture experiments with dissociated *Xenopus* spinal neurons have shown that neuronal responses to netrin-1 could be converted between attraction and repulsion by altering the cAMP level in the growth cone (Ming et al., 1997). Elevated levels of cGMP can also change the response of growth cones to a semaphorin from repulsion to attraction (Song et al., 1998). Remarkably, an asymmetric cellular localization of sGC to the dendrite of pyramidal cells is thought to confer the opposite directional outgrowth to dendrites and axons in a semaphorin gradient of the cerebral cortex (Polleux et al., 2000). Thus, intracellular cyclic nucleotide levels can be the critical factors that govern process extension to the same chemotropic guidance cue.

In non-neuronal cells, the influence of cGMP/cAMP signalling on cell migration is well studied. In primary cultured aortic smooth muscle cells, NO/cGMP stimulates migration, regulates cell morphology and cytoskeletal organization (Brown et al, 1999). Whereas, cAMP donors and activators of PKA exhibit an inhibitory effect on migration on smooth muscle cells (Sun et al., 2002). Similarly, the migration-stimulatory effects of cGMP were also found on macrophages, endothelial cells and epithelial cells (Elferink and Van Uffelen, 1996; Ziche et al., 1994; Noiri et al., 1996). In postcapillary endothelial cells, inhibition of NOS attenuates cell migration. NO/cGMP signalling induced a switch from a stationary to a locomoting endothelial cell type (Ziche et al., 1997). In the present study NO/cyclic nucleotide mediated neuronal migration is described for the first time.

4.3. NO/cGMP signalling serves as permissive factor for MG neuron migration

Cell surface, extracellular matrix, and diffusible signalling molecules including netrins, Slits, semaphorins, and ephrins have been implicated as major cues in axonal guidance (Tessier-Lavigne and Goodman, 1996; Mueller, 1999; Song and Poo, 2001; Yu and Bargman, 2001; Dickson, 2002). These extracellular signals have to be interpreted by the intracellular signal transduction machinery that drives the cytoskeletal rearrangements leading to directed fibre outgrowth. A recently emerging theme in neuronal development is that axon guidance cues can also guide neuronal migration (Song and Poo, 2001). The main difference between neuronal migration and axonal navigation is the directed movement of the cell body. Thus it is not unlikely, that mechanisms similar to growth cone guidance may also

operate during the unidirectional translation of cell bodies. For example, the secreted protein Slit plays a role in repelling the projections of axons from the midline in *Drosophila*. (Battye et al., 1999; Kidd et al., 1999). But Slit has also been shown as a guidance cue for neural precursors migrating from the subventricular zone to the olfactory bulb (Wu et al., 1999), the migration of extracortical GABAergic neurons into the embryonic neocortex (Zhu et al., 1999), and the correct ventral positioning of inferior olivary neurons (Causeret et al., 2002). Thus, the findings that NO is a permissive regulator of pioneer axon extension in the grasshopper embryo (Seidel and Bicker, 2000), motivated me to explore the role of NO/cGMP signalling in the context of neuronal cell migration. Pharmacological manipulations of NO/cGMP levels in intact insect nervous systems have also uncovered functional involvement of cGMP in the growth cone behaviour of *Drosophila* photoreceptor projections (Gibbs and Truman, 1998) and of grasshopper pioneer neurons (Seidel and Bicker, 2000). Here, I reported that NO induced cGMP production is a permissive signalling pathway for the migration of enteric neurons on the midgut of a simple invertebrate preparation. These data suggest that the MG neurons may receive a NO signal from the visceral midgut cells and that elevated cGMP levels are essential for the ability of migration. Could a tissue-intrinsic NO signal play a role as a guidance factor for the cell migration? Diaphorase staining appears to be distributed in all areas of the midgut surface (Fig. 4E) and is not exclusively confined to cells in the vicinity of the four migratory pathways. Therefore I view it as rather unlikely that NO release can prefigure the migratory routes on the midgut.

Alternatively, the midgut cells may synthesize NO in an anterior/posterior gradient which might provide directional information. Since I have not found any significant misrouting by the MG neurons after pharmacological inhibition of NOS or sGC (Fig. 14), there is no evidence for a directional guidance function of NO. This view receives support from transplantation experiments in the ENS of *Manduca* showing that EP cells are capable of migrating in both directions along the host muscle band (Copenhaver et al., 1996).

To fully appreciate the role of NO/cGMP signalling in cell migration, it is essential not only to investigate the spatial distribution of NO synthesizing cells, but also to monitor the temporal pattern of NO/cGMP formation and breakdown. Similar to the vertebrate nervous system, neuronal production of NO in the locust is a tightly Ca^{2+}/calmodulin regulated process (reviewed in Bicker, 1998). Thus, increases in

cytosolic Ca^{2+} levels may provide a developmental timing signal for the production of NO. In MG neurons, the resulting changes in cyclic nucleotide levels will then allow for the transduction of extracellular guidance cues to the rearrangement of the cytoskeletal elements in motile cell regions. The pharmacological blocking of cell migration by RPcGMPS (Fig. 6) points towards a functioning of PKG in cell motility downstream of NO/cGMP signalling. The initial appearance of inducible sGC activity in the MG neurons just at the onset of migration suggests that NO/cGMP signalling might be required for the initiation of migratory behavior. In primary cultured aortic smooth muscle cells, NO induces changes in cell shape, reorganization of the cytoskeleton and reduction of adhesion (Brown et al, 1999). Correspondingly, in the grasshopper ENS, NO might be required as a permissive factor for the initiation and maintenance of MG neuron migration.

The hypothesis that cyclic nucleotides and their effector enzymes mediate neuronal migration gets further support by the findings that cAMP and PKA signalling has inhibitory effects on MG neuron migration. To regulate cell migration, protein kinase activation will ultimately have to cause cytoskeletal rearrangements. The results of this study indicate, that NO/cGMP/PKG signalling affects the actin cytoskeleton. In vertebrates, several lines of evidence suggest that proteins of the Enabled (Ena) and vasodilator-stimulated phosphoprotein (VASP) protein family may be involved in directly regulating actin polymerization (Lanier and Gertler, 2000). The Ena/VASP proteins are major substrates for cyclic nucleotide-dependent kinases in platelets and other cardiovascular cells. They promote actin nucleation and bind to actin filaments *in vitro* and associate with stress fibres in cells. Phosphorylation significantly alter the actin binding properties of VASP (Harbeck, 2000,JBC) Moreover, in human neutrophils PKG influence neuronal migration by remodelling the actin cytoskeleton via phosphorylating one of its substrates, the actin-organizing protein ENA/VASP (Reinhard et al., 2001). However, though all vertebrate Ena/Vasp proteins are PKA targets, invertebrate Ena does not appear to contain sites for PKA phosphorylation (Lanier and Gertler, 2000). Another possible link between PKA and actin may be Spinophilin, a protein phosphatase 1 - and actin-binding protein that modulates excitatory synaptic transmission and dendritic spine morphology. Phosphorylation of spinophilin by PKA modulated the association between spinophilin and the actin cytoskeleton.

The actin cytoskeleton is critical for the establishment of cell shape and motility (Hall, 1998). Key players in the regulation of the actin cytoskeleton are members of the Rho family of small GTPases, which have emerged as the principal transmitters of signals from transmembrane receptors that stimulate actin filament nucleation (Bishop and Hall, 2000). The most extensively characterized members are Rho, Rac and Cdc42, which control the formation of actin stress fibres, lamellipodia and filopodia, respectively. These distinct actin-remodelling events are a consequence of the selective interaction of the activated Rho GTPases with specific effector proteins linked to the actin cytoskeleton. For example, RhoA can be inhibited by phosphorylation through PKA, which correlates with an inhibition of lymphocyte motility by cAMP (Lang et al., 1996, EMBO). In vascular smooth muscle cells, PKG phosphorylates and inhibits RhoA which leads to a reorganization of the actin cytoskeleton (Sauzeau et al. 2000, JBC). However, the complete molecular mechanisms that link PKG or PKA signalling to the actin cytoskeleton remains to be elucidated.

The findings of this paper together with the study of pioneer neuron outgrowth (Seidel and Bicker, 2000) implicate NO/cGMP signalling both in axon elongation as well as in neuronal migration in the grasshopper. Since growth cone and cell migration can be visualized and experimentally manipulated in the intact embryo, this accessible system will thus allow to elucidate further molecular steps of the cyclic nucleotide signalling pathways on cytoskeletal rearrangements.

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